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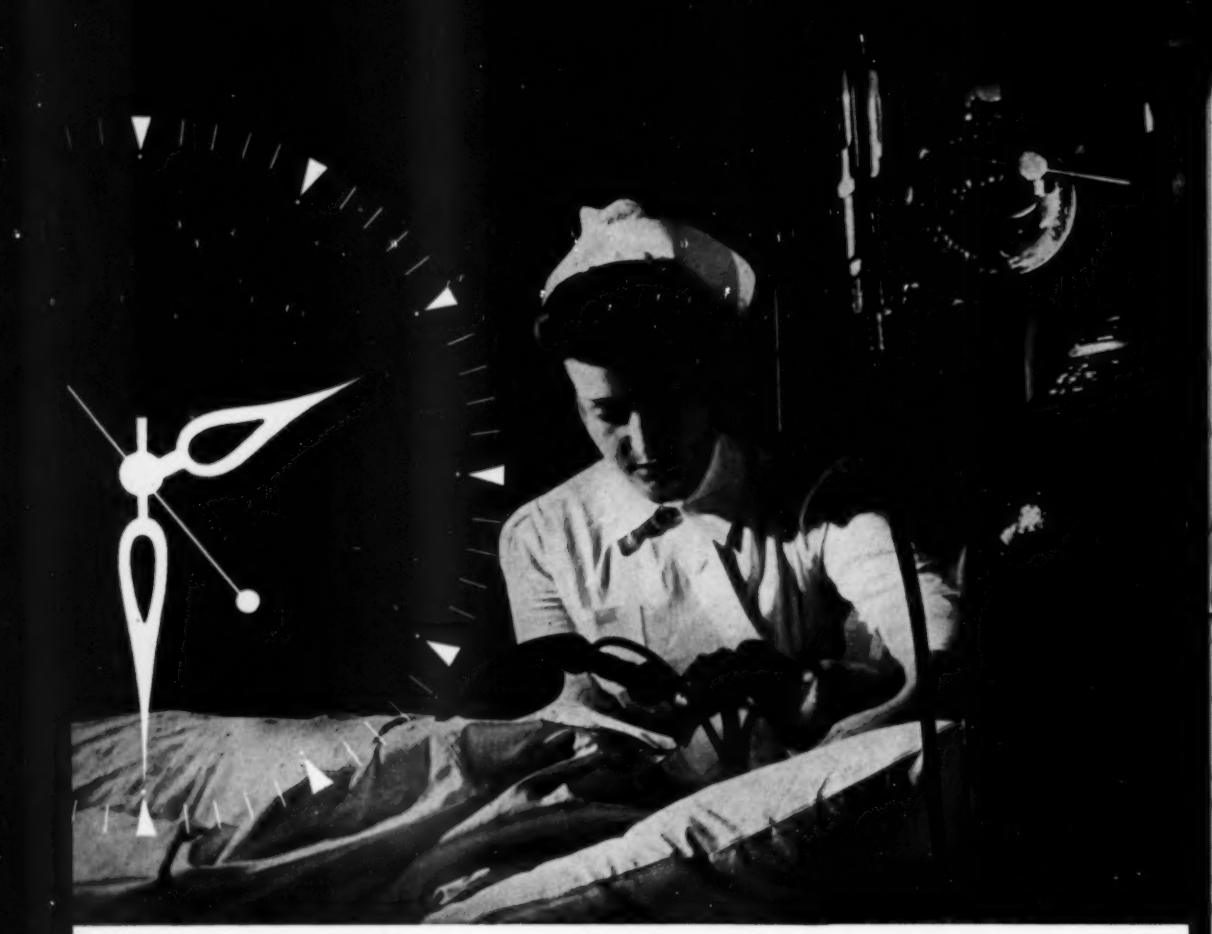

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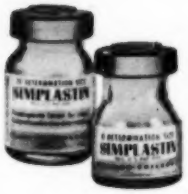

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ARCHIVES OF PATHOLOGY

VOLUME 57 JUNE 1954 NUMBER 6

SOME PITFALLS IN THE PERFORMANCE AND THE INTERPRETATION OF RESULTS OF SEROLOGIC TESTS FOR VIRUS AND RICKETTSIAL DISEASES

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THERE are two major approaches to the laboratory diagnosis of viral and rickettsial diseases, as there are for all the diseases of microbiological origin. These are (*a*) the direct isolation and identification of the causative agent and (*b*) the indirect identification of the agent by performing tests for the detection of antibody.

The direct methods are expensive, time-consuming, and often dangerous.[1] The opportunity for obtaining the etiological agent may have passed by the time the physician is called to see the patient,* since many viruses are present in body fluids or blood only during the incubation period. It is also well known that inoculation of animals or chick embryos with sterile material may activate latent viruses which naturally occur in these animals, and the consequent infection could erroneously be considered as being related to the patient's illness.† Moreover, few laboratories are properly equipped to carry on routine isolation of viruses and rickettsiae.

At present, the indirect or serologic methods for laboratory diagnosis of the virus and rickettsial diseases have a greater usefulness than those which involve direct isolation of the specific disease agents. Among the important serologic tests are the neutralization test, the heterophile antibody test of Paul and Bunnell,[6] the hemagglutination inhibition test, the cold hemagglutination test, the Streptococcus MG agglutination test, the complement-fixation test, and the complement-fixation inhibition test. Certain of these tests can now be carried out in the large hospital or state public health laboratory on a routine basis. They will be of value, however, only if the proper methods are utilized and correct interpretations are made from the results obtained. The discussions which follow will make these statements clear.

When tests which measure antibody are employed for laboratory diagnosis, it must be realized at once that a single test can seldom give the physician the answer he is seeking. This is because a single test of this type usually cannot establish whether the antibody present is due to previous subclinical experience with the disease, previous active disease, present active disease, previous vaccination, or an anamnestic response. Also, it must be remembered that because of the wide varia-

From the Department of Bacteriology and the Department of Pathology, Northwestern University Medical School.

* Rhodes,[2] p. 111.

† References 3-5.

tion of antibody response in different individuals a negative serologic test does not always rule out a given disease. It follows, therefore, that a series of tests must be employed to demonstrate a definite rise in titer. Two or three specimens of sera are drawn and examined at suitably spaced time intervals. The significance of this rise must be evaluated in the light of several factors, including the nature of the disease under consideration, the time of onset of the patient's illness, the day of collection of the specimen, the method of handling the specimen, and the temperature at which it has been stored since its withdrawal. These data, plus a concise statement of the patient's history, must be made available to the virologist if the physician desires that he interpret this type of test for him. The senior author reports the results of tests done in his laboratory together with a written interpretation in a manner similar to that currently employed by most pathologists for surgical specimens.[7]

It is the purpose of this communication to indicate briefly the principles concerned in each of the above methods, to outline some of the problems involved in the performance of the tests, to discuss the problems of the interpretation of results obtained, and to indicate the usefulness of the test in routine laboratory diagnosis.

TABLE 1.—*Results of Neutralization Test with Unknown Serum**

Dilutions of Virus	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Poliovirus alone	10:10†	7:10	5:10	0:10
Poliovirus and 1:5 unknown serum	3:10	3:10	1:10	0:10
Poliovirus and normal monkey serum	10:10	8:10	5:10	1:10
Poliovirus and 1:5 known immune serum	2:10	2:10	1:10	0:10

* Observation period 35 days.
† Mortality ratio 10:10 means 10 animals died out of 10 inoculated.

THE NEUTRALIZATION TEST

In the neutralization test, mixtures of virus-infected tissue and test serum are prepared. These mixtures are then incubated for varying lengths of time and at varying temperatures, depending upon the virus concerned, and then the mixture is injected into a series of animals. The amounts of virus-infected tissue used may be kept constant and serial dilutions of serum added, or the amount of serum employed may be kept constant and the virus-containing material diluted. For each dilution used, a series of 5 to 10 laboratory animals or chick embryos is injected and the animals are kept under observation for a number of days. The number of deaths occurring in each group of animals is recorded. These results are then compared with the ability of the virus to kill animals when injected without adding serum. If one is searching for antibody in a serum from a patient, and it is present in the above mixtures, only the more concentrated amounts of virus will be able to kill the animals. If one is employing the test to identify an unknown virus, the known antibody-containing serum should be able to inhibit the killing power of the unknown virus to approximately the same degree as it does the known strain of virus from which it was prepared. Table 1 presents an example of a neutralization test in which an unknown serum is shown to contain antibody against the type II strain of the virus of poliomyelitis. There are a number of reasons why this test is unsatisfactory for routine use. A stock virus must constantly be maintained by animal passage. Test animals in considerable numbers must be on hand also. (These two

facts alone make the test too costly for the average laboratory.) A considerable period of time must elapse before a neutralization test is completed. When the test is employed with the neurotropic viruses, this may be a matter of from four to six weeks.[8] Furthermore, repeated specimens must be tested in order to demonstrate a rise in titer, which increases the time as well as the expense.

One of the most important things to remember in interpreting the results from neutralization tests is the wide variation in the time of appearance of antibodies following the onset of various virus diseases. Antibodies against influenza may appear within 48 hours after onset, whereas those against lymphocytic choriomeningitis may not be present until 8 to 10 weeks after the acute stage of the disease.[9] However, a negative neutralization test on a serum specimen taken two months after onset has a clear-cut significance in most instances. We wish to confirm Milzer's [1] suggestion that the neutralization test is very useful when employed as a check on positive complement-fixation tests for the neurotropic viruses.

The recent advances in tissue culture technic for use in the laboratory diagnosis of poliomyelitis have added greatly to the methods available for basic research and epidemiological studies in relation to this disease. Neutralization tests may be performed in which growing cells of the monkey kidney or human embryo in culture are substituted for living laboratory animals.‡ While these procedures are far superior to the use of monkeys or other laboratory animals, they are not suitable for routine laboratory diagnosis at present. Perhaps the stimulus of these recent studies will result in such improvements that the neutralization technic will become more adaptable to use by the average laboratory for a number of virus diseases.

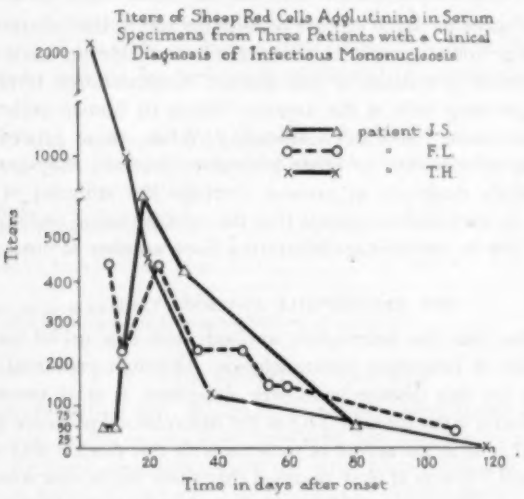
THE HETEROPHILE ANTIBODY TEST

It is well known that the heterophile antibody test is a useful method for the laboratory diagnosis of infectious mononucleosis. Although protozoal,[12] bacterial,[13] and viral [14] causes for this disease have been described, it is at present most frequently considered as a virus disease. The as yet unexplained presence of agglutinins for sheep red blood cells in the serum of patients with this disease was demonstrated by Paul and Bunnell.[6] It was at first assumed that these antibodies were Forssmann in nature, and many physicians will speak of (or order) the test by that name. Bailey and Raffel [16] and subsequently others showed that the sheep red cell agglutinins appearing in infectious mononucleosis differed from similarly acting agglutinins in serum sickness (Forssmann) and normal serum. This difference usually can be demonstrated by an absorption technic reported by Davidsohn [17] employing cooked beef red blood cells and guinea pig kidney antigens as follows: The agglutinins found in normal serum are absorbed by guinea pig kidney but not by beef cells. The agglutinins occurring in serum sickness are absorbed by both antigens, while the antibodies of infectious mononucleosis may be only slightly reduced by absorption with guinea pig kidney but are absorbed by beef cells.

In most instances the absorption tests have proved to be satisfactory, but occasionally inconsistent results have been observed. Wechsler [21] reported in 1946 that the absorption tests with guinea pig kidney and beef red cell suspensions did not give uniform results in six of a series of 256 cases. Demanche [22] indicated that in one instance (in a case of infectious mononucleosis) there was no absorption by

‡ References 10 and 11.

either antigen, while in another the affinity of the beef cells for the antibody was only demonstrable after 24 hours of incubation. In yet another recent report it was brought out that agglutinins from patients with infectious mononucleosis have given the reaction usually reported for normal serum, while, in a further instance, serum from a patient with serum sickness gave the reaction characteristic for infectious mononucleosis.[23] These data do not mean that the absorption tests should not be performed; in fact, this information, when considered together with the report that sheep red cell agglutinins have been found in conditions other than infectious mononucleosis, should make the absorption test imperative. Sheep red cell agglutinins are found in primary atypical pneumonia, infectious hepatitis, German measles, scarlet fever, polycythemia vera, tuberculosis, monocytic leukemia, Hodgkin's disease, and following the injection of horse serum.[24] The use of the absorption methods and a careful history will in most instances eliminate these cases from consideration.



It is now evident that the determination of a titer by a single test, by somewhat different technics and with different batches of sheep red blood cells, has much less significance than is generally accepted.§ Sheep red cell antibodies in this disease do not appear before the fifth day of illness.‖ The peak of the antibody level is attained in the majority of instances approximately within the first month, and may reach a level as high as 1:229,000. Titers of 1:1,000 to 1:5,000 are not uncommon in the acute phase of the disease. Although on the average antibodies persist for 80± days, a case has been reported in which these substances were found 309 days after onset.‖ An occasional patient is encountered in which the test does not become positive until three to six months after onset.¶

§ Reference 18; Harding, H. B., unpublished data.

‖ Leibowitz,[19] p. 89.

¶ Reference 20. Davidson, I.; Stern, K., and Kashuvagi, C.: Notes from Infectious Mononucleosis Exhibit, 27th Annual Meeting of the American Society of Clinical Pathologists, Chicago, Oct. 12-15, 1948.

The Chart presents a record of titer levels from three patients with a clinical diagnosis of infectious mononucleosis. Notice the wide variation in immune response among these few patients. If a number of specimens had not been examined from the patient J. S., the diagnosis might have been missed entirely. Perhaps the somewhat confusing picture of the application of the heterophile test to the diagnosis of infectious mononucleosis will be clarified by reviewing the following points:

1. A series of tests is superior to a single test, and it is desirable to demonstrate a rise in titer.
2. No single titer level is always diagnostic. Low titers which persist after proper absorption and rise during infection may be as significant as higher titers.
3. If the titer level is 1:2,000 or better in the absence of a suspicion of serum sickness or horse serum injection, it may for practical purposes be considered diagnostic for infectious mononucleosis. Titers of a lower order must be confirmed by absorption tests.#
4. Inconsistent results occasionally are obtained with the absorption tests, and heterophile antibodies may occur in other diseases.
5. Since a standard procedure is of utmost importance, it is suggested that the techniques employed by Davidsohn* be universally adopted.

VIRUS HEMAGGLUTINATION AND THE HEMAGGLUTINATION INHIBITION TEST

In 1941, Hirst [25] first observed that when virus-infected allantoic fluids became mixed with chick embryo blood the erythrocytes firmly agglutinated. Adult chicken red cells, washed with saline, also were agglutinated by virus-containing fluid. From this work the virus hemagglutination reaction (VHA) was developed as an accurate means of titrating virus in previously inoculated chick-embryo fluids. The next step which followed was the development of the virus hemagglutination inhibition (VHI) test. In other words, antibody-containing serum when introduced into a system of homologous virus and susceptible erythrocytes will inhibit agglutination of the red cells by the virus in proportion to the amount of antibody contained in the serum. This latter type of test can be employed to identify virus using a known serum or to determine the titer and type of antibody in an unknown serum when known viruses are employed.

The virus hemagglutination inhibition reaction is most frequently used in the diagnostic laboratory to measure the presence or absence of antibody and thus to aid in diagnosis by the indirect approach. The test has been successfully employed with viruses of the influenza group, S-K poliomyelitis virus, Theiler's virus, mumps virus, the virus of Newcastle disease of fowl, and the virus of fowl plague. The red cells of human group O and erythrocytes of guinea pigs and sheep have been tried by various workers against different viruses in this reaction. The test has had its greatest application, however, in relation to the routine diagnosis of influenza. The most useful form of the test is known as the "pattern" test as described by Salk [26]; it employs chick red blood cells. The "pattern" test has the following advantages: (*a*) ease of performance; (*b*) sharp, easily interpreted end-points; (*c*) no necessity for special equipment; (*d*) wide variance in the amount of virus which may be employed without adversely affecting the test; (*e*) a permissibly wide variation in incubation time before reading; (*f*) economy.

Leibowitz,[19] p. 113.

* Davidsohn, I.; Stern, K., and Kashuvagi, C.: Notes from Infectious Mononucleosis Exhibit, 27th Annual Meeting of the American Society of Clinical Pathologists, Chicago, Oct. 12-15, 1948.

In preparation for the inhibition test, it is first necessary to determine the titer of virus contained in a pool of allantoic fluid removed from chick embryos previously inoculated with an influenza virus. Several strains are now usually used and are prepared independently. These include type A (PR 8), type A prime (FM-1, Cuppett) type B (Lee), type C (1233). The highest dilution of the virus-containing fluid which will give complete agglutination of a 0.25% concentration of chick red blood cells in saline is determined. This is called the "agglutinating" unit. In the inhibition test, a constant amount of virus (four agglutinating units) is added to falling twofold dilutions of serum. After the serum and virus are mixed, a constant amount of 0.25% concentration of chick red cells is added to each tube in the series and the tests are allowed to stand at room temperature for one and one-half hours. In tubes without agglutination the cells settle into the bottom of the tube in a small, compact, sharply-outlined, button of cells, while with positive agglutination the cells settle in a diffuse disc with serrated edges. By this method fourfold or greater titer rises between acute- and convalescent-phase sera, examined on the same day against the same red cell suspension and against the same antigen dilution, are considered significant evidence of influenza infection.

Human infection with influenza virus may result in an antibody response which ranges from no detectable change to a 100-fold or greater increase in circulating antibody level.[27] In influenza epidemics or in sporadic cases there are usually more subclinical infections than clinically manifest ones and, unfortunately, there is no difference in mean antibody response between these two groups. The percentage of positive serological results obtained in typical clinical cases varies with the epidemic, the type and strain of virus involved, and the preepidemic antibody level of the population. An average figure would probably be 60 to 75% of the cases tested,

It is not uncommon to find antibody response to influenza antigens other than the type causing the infection, since there are shared antigenic components among all the influenza viruses. This fact makes the determination of the type of agent causing the infection a difficult task at times.

In certain epidemics it has been necessary to isolate the particular strain of virus concerned and to employ it as an antigen before serologic tests on specimens from involved persons could be properly interpreted.[27] It should now be clear that a negative serologic test does not necessarily rule out infection. It is also possible that an antibody rise may not reflect clinical influenza but an anamnestic response, particularly if it fades rapidly. Many persons now carry antibodies to influenza viruses gained through previous vaccination. Thus, in each instance the results obtained must be carefully interpreted in the light of the patient's clinical history.

THE COLD HEMAGGLUTINATION TEST AND THE STREPTOCOCCUS MG AGGLUTINATION TEST

Arrasmith [28] was among the first of a series of workers to call attention to certain pneumonias, occurring both sporadically and in small epidemics, which failed to conform to the accepted picture of either lobar or bronchopneumonia. In a number of instances the agents of psittacosis-ornithosis, influenza, Q fever, lymphocytic choriomeningitis, and tularemia were found to be the cause. On the whole the cases caused by these agents were severe. There remained a larger group of unproved etiology which were milder. These cases had been variously labeled "acute pneumonitis," "virus pneumonia," and "primary atypical pneumonia." Despite many

thorough investigations, conclusive evidence of a single etiologic agent is still lacking.[29] Recent observations that chlortetracycline (Aureomycin) therapy exerts a favorable influence on the course of the disease lends support to the view that a member of the psittacosis-ornithosis group of basophilic viruses is concerned.† Weir and Horsfall [30] and others have implicated at least five different filterable agents. Though we still lack the ability to demonstrate the etiologic agent, there are two serologic tests, quite independent of each other, which are useful adjuncts to the diagnosis of this disease. The cold hemagglutination test is the most important of these two methods. This test is based on the observation by Clough and Richter [31] that serum from a patient whose case they had diagnosed as "bronchopneumonia" contained agglutinins for human group O erythrocytes. These are operative at temperatures ranging from 0 to 10 C. At 37 C., the reaction disappears. Peterson and others [32] have established that this phenomenon occurs in primary atypical pneumonia. For the test, blood specimens should be obtained from the patient as early in the acute phase of the disease as possible and weekly thereafter until a diagnosis is made. It is very important that the blood be allowed to clot at room temperature and the serum be removed before it is stored in the icebox. This is necessary because of the fact that agglutinins in the serum will adsorb onto the patient's own red blood cells if the blood is placed immediately in the icebox. Twofold descending serial dilutions of the serum are prepared in 0.85% salt solution. To each tube a standard amount of a 0.2% saline suspension of thoroughly washed group O human erythrocytes is then added. After thorough shaking, the rack of tubes is kept in the icebox at approximately 4 C. overnight. The following morning the tubes are removed from the icebox, one tube at a time, and read for the degree of agglutination. The greatest amount of agglutination is characterized by a tight button of red cells which does not easily break up on gentle shaking (4+) and grades down to fine agglutination observable only through the low power of a microscope (1+). Positive tests must again be read for the disappearance of agglutination after 30 minutes at 37 C. to eliminate the presence of heterophile agglutinins.

Cold agglutinins appear at the end of the first week of illness and usually reach their maximum in two to four weeks, declining in four to six weeks. Feller [33] states that high titers (1:128 to 1:1,024) are not often seen except in primary atypical pneumonia and certain hemolytic anemias. Cold agglutinins of a lower order have been described in trypanosomiasis, blackwater fever, mumps orchitis, certain liver disorders, and peripheral vascular disease, however. Experience has shown that primary atypical pneumonia is the only respiratory disease which is likely to lead either to a large increase in titer in serially examined sera or to the presence of high titer cold agglutinins in a single specimen taken at the proper time after onset of illness. Of course, demonstration of a rising titer in serially drawn sera is the most convincing evidence. Fourfold or greater increases are significant. Also, a definite decrease in titer six to eight weeks after onset is important corroboratory evidence. It should be emphasized that the failure to demonstrate cold agglutinins does not necessarily exclude the diagnosis of primary atypical pneumonia. Cold agglutinins have been reported in from 20 to 90% of the cases, depending upon the time of sampling and the severity of the disease in the given patient.[29] This test can be performed in the average hospital or public health laboratory.

†Rhodes and Van Rooyen,[2] p. 231.

Streptococcus MG is a nonhemolytic Streptococcus which appears to be distinct serologically from other species of streptococci. It is distinct from, though immunologically related to, Streptococcus salivarius Type I.[33] Mirick and Thomas [34] isolated this micro-organism from the lungs of persons with fatal attacks of primary atypical pneumonia and suggested that it was possibly implicated in the cause of the disease. It has, however, been found in the upper respiratory tract of normal persons. About 50% of persons with primary atypical pneumonia develop agglutinins to this organism. Rivers [35] suggests three possible explanations for this phenomenon: First, there may be a coincidental immunologic relationship between the bacterium and some other infectious agent (a heterophile response); second, the reaction may be due to a secondary invasion by the micro-organism; third, the agglutination may result from a double infection initiated by the Streptococcus and some other infectious agent, presumably a virus (such as occurs in swine influenza).

Several specimens of serum should be tested, beginning in the early acute phase and continuing on a weekly basis until the diagnosis is made. The antigen is a heat-killed, washed suspension of an 18-hour broth culture of the organism adjusted to No. 5 on the McFarland scale, to which thimerosal (Merthiolate) in a final concentration of 1 : 10,000 is added as a preservative.

Serial descending twofold dilutions of unheated serum are prepared in isotonic saline solutions. An equal volume (0.5 ml.) of antigen suspension is added to each tube. The test is incubated in a water bath at 37 C. for two hours, followed by incubation overnight in the icebox at 4 C. The rack is again placed in the water bath at 37 C. for two hours, after which the tubes are shaken and read. The second period of 37 C. incubation is very important, since nonspecific agglutination of Streptococcus MG in the icebox may occur.

Antibodies to this Streptococcus usually do not begin to appear in the serum of patients suffering from primary atypical pneumonia until the second or third week after the onset of illness and usually reach maximum levels during the fourth and fifth weeks. As is the case for cold agglutinins, the antibody level is correlated with the severity of illness. The test has been reported positive in about 50% of persons suffering from primary atypical pneumonia. Maximum titers are not strikingly high in this test. In Horsfall's [36] series, only 2.5% of cases gave a titer higher than 1 : 160. Titers of 1 : 10 or 1 : 20 must be interpreted with caution. The demonstration of a fourfold or greater rise in titer during convalescence is considered significant. Such a rise has almost never been demonstrated against Streptococcus MG except in primary atypical pneumonia. Low titers of agglutinins without the rise have been demonstrated in persons with various acute respiratory infections, in normal persons, and in persons with certain localized streptococcal infections.

Both the cold hemagglutination test and the Streptococcus MG test are of value only in making a retrospective diagnosis. They therefore offer little to the clinician during the acute phase of the disease when various forms of therapy are being considered. This does not, however, relieve the physician of his responsibility for making an etiological diagnosis.

THE COMPLEMENT-FIXATION TEST

The complement-fixation test, as applied to the laboratory diagnosis of viral and rickettsial diseases, has had a remarkable development within the past five to six years. The problem has been to prepare antigens which were noninfectious,

stable, sensitive, specific, and commercially available. Though further improvement in specificity and reliability are needed, the antigens which are now commercially available ‡ are sufficiently satisfactory so that they may be employed in routine laboratory diagnosis. We have employed this method with sera from hospital and clinic patients for more than three years [7] and have found it to be the most satisfactory routine diagnostic method presently available for a large teaching hospital. The method has the following advantages in addition to the availability of antigens:

1. A technician who is competent to perform the various complement-fixation techniques used for the laboratory diagnosis of syphilis can, with proper additional training, also perform these tests.
2. The same equipment needed for the above tests also can be employed for these tests.
3. The hemolytic system reagents which are prepared and standardized for the former method can be used for the latter.
4. Large numbers of laboratory animals need not be maintained. The required guinea pig complement and sheep red blood cells can be obtained weekly on a commercial basis. Rabbit serum hemolysin can be purchased and stored for long periods of time.
5. Complement-fixing antibodies appear in the patient's serum relatively earlier in the course of the disease than do neutralizing antibodies except in the case of equine encephalomyelitis.[37] Therefore the test is more useful in day-to-day laboratory work than the neutralization test.
6. As a diagnostic method this test is much less time-consuming and less expensive than the neutralization test for those diseases to which it can be applied.

The complement-fixation technique can be routinely used for the following diseases: influenza (types A, A′, B, and C), psittacosis-human pneumonitis, Q fever, Western equine encephalitis, Eastern equine encephalitis, Japanese B encephalitis, rabies, mumps, lymphogranuloma venereum, Rocky Mountain spotted fever, murine typhus, epidemic typhus, rickettsialpox, herpes, and variola. No complement-fixation tests are available for primary atypical pneumonia, the common cold, herpes zoster, German measles, measles, chicken pox, inclusion conjunctivitis, epidemic keratoconjunctivitis, infectious hepatitis, infectious mononucleosis, and homologous serum jaundice. Although complement-fixation methods are available for the Coxsackie group of viruses, there are so many immunologically slightly different viruses in this category and antibodies to these agents are so widespread in the adult population of the world, that it is almost impossible to interpret findings when they are obtained.§ The complement-fixation technique is now being successfully applied to the laboratory diagnosis of poliomyelitis in research laboratories by employing antigen prepared from tissue cultures of the etiologic agent,[38] but it has not been developed to the point where it is of routine usefulness in the diagnosis of the disease.

Antigens which are in use for virus and rickettsial diseases consist of suspensions of infected tissue which are prepared and/or extracted in various ways. The chick embryo, the mouse, and the guinea pig are the laboratory hosts from which the infected material is obtained for their manufacture.[1] Soluble components of the influenza and mumps viruses and certain of the rickettsiae have been shown to

‡ Markham Laboratories, Chicago 20; Lederle Laboratories Division, American Cyanamid Company, New York 20; E. R. Squibb & Sons, 745 Fifth Avenue, New York 22; Parke Davis & Co., Detroit.

§ Huebner, R. J.: Personal communication to the authors.

make useful antigens.|| Improved methods of extraction have largely eliminated the anticomplementary properties often encountered in earlier viral and rickettsial complement-fixation antigens. Since virus-containing tissue suspensions are employed as antigens, similarly prepared and extracted normal tissue suspensions must be used as controls.

For examination by this method, a specimen of at least 20 ml. of blood should be obtained under aseptic conditions from the patient. This amount of sample is needed because most laboratories perform a battery of complement-fixation tests on each specimen. In our work, each specimen is tested against 16 different antigens. Since it is possible that some of the same specimens may be employed in the cold agglutinin test, we routinely allow the specimen to clot at room temperature and remove the serum before it is stored. Hemolized serum and high-lipid-containing serum are unsatisfactory specimens. Therefore a fasting specimen drawn into a sterile container without anticoagulant should be obtained. The whole-blood specimen should never be frozen before the serum is removed. It is of utmost importance

TABLE 2.—*Optimal Time for Obtaining Serum Specimens for Complement-Fixation Tests on Various Viral and Rickettsial Diseases*

Disease	Acute Phase Specimen	Convalescent Phase Specimens
Influenza	Before 3d day	10th day and after
Psittacosis	Before 10th day	After 21 days
Lymphogranuloma venereum	Before 10th day	After 21 days
Encephalitis group	Before 3d day	After 14 days*
Lymphocytic choriomeningitis	Before 10th day	After 21 days
Rickettsial group	Before 6th day	After 12 days
Mumps	Before 6th day	After 12 days
Rabies	Before 3d day	.....
Herpes simplex	Before 5th day	After 14 days
Variola	Before 5th day	After the 10th day

* One-third between 30 and 60 days.

that each specimen be accompanied by a concise history of the illness, stressing date of onset, clinical findings, history of recent travel, history of previous viral illnesses, and history of previous vaccinations and/or inoculations.

As in the case for the previous serologic methods discussed, a laboratory diagnosis is seldom if ever made on the basis of the examination of a single specimen. Many persons carry antibodies to various viral and rickettsial diseases in their serum as the result of a previous inoculation or previous infections, clinical or subclinical. Though in general the antibody level tends to fade out as time passes, in certain instances a high level of antibody may be maintained for years. It is also known that the time following onset at which antibodies appear varies widely with various virus diseases. Table 2 [41] gives examples of the best times for obtaining serum specimens for certain virus and rickettsial diseases.[47] After a study of this table it can be seen that it is imperative to examine at least two specimens to insure that interpretable results will be obtained.

The principle of this test is the same as that which governs the methods in use in syphilis serology. The suspect serum is first inactivated. (In our work we have used 57 C. for 30 minutes. We have found this superior to employing 60 C. for 20

|| References 39 and 40.

minutes as Milzer has suggested.[1]) Adequate inactivation is necessary in order to reduce the activity of certain nonspecific substances and to destroy native complement. In order to examine large numbers of specimens, a screening procedure is used. The patient's serum is diluted in twofold descending serial dilutions from 1:4 through 1:64 for the antigens of lymphogranuloma venereum, psittacosis, the influenzas, and mumps. The specimen is set up in dilutions of 1:4, 1:8, and 1:32 for the antigens of rabies, Eastern equine encephalitis, Western equine encephalitis, St. Louis encephalitis, Japanese B encephalitis, lymphocytic choriomeningitis, and the rickettsial antigens. Sera which are found positive in the screening test are retested against the reacting antigen to determine the end-point titer. One unit of antigen (0.1 ml. of a 1:4 dilution) is mixed with two full units of complement (0.2 cc.) and 0.2 ml. of each serum dilution. The mixture is then incubated for 80 minutes in a water bath at 37 C. The hemolytic system is then added; it consists of 0.4 ml. of equal volumes of 2% suspension of sheep cells and hemolysin (ambocepter) solution containing two hemolytic units. After further incubation for one-half hour at 37 C. or until the complement control has cleared, the tests are read. If samples of serum cannot be tested shortly after they are drawn, they may be stored in the icebox in screw-capped plastic-washered Kimble glass tubes for periods up to two weeks.[42] After this time the titer drops. If they are to be stored for longer periods they must be flame-sealed in chemically clean glass ampules and stored in the icebox to prevent rapid drop in titer. Positive control sera should be similarly stored in sealed glass ampules.

While it is true that any laboratory capable of making satisfactory routine complement-fixation tests for syphilis also has the facilities for conducting viral and rickettsial complement-fixation studies, it must be a laboratory which performs sufficient numbers of these tests at regular intervals. This is the only way that one can learn about the minor difficulties incident to the performance of virus complement-fixation tests and what to do about correcting them. The persons who perform these tests should be adequately trained in the special techniques which are employed.

A fourfold rise in titer is the minimum rise which is accepted by most authorities as indicative of present active disease. There are certain exceptions to this rule, however, which must be carefully interpreted in relation to the patient's history. If the first specimen examined shows a high titer and the history indicates that the result obtained is about what would be expected at this stage in the patient's illness, one may make a presumptive serological diagnosis. If on testing a second specimen, a drop in titer is recorded which corresponds to the fading curve of the antibody level for the disease in question, then the diagnosis can be considered confirmed.

The dependence upon a fourfold titer rise alone without consideration of the patient's history, clinical findings, and other laboratory data may lead to an erroneous diagnosis, as the following example will illustrate: We [43] recently investigated an epidemic of primary atypical pneumonia. Fourfold titer rises for influenza B antigen were obtained after serial specimens were drawn (Table 3) from three of the patients (data on two patients given in table) early in the outbreak. As can be seen from the data in Table 4, there can be little doubt that these persons were suffering from primary atypical pneumonia and not influenza type B. Since these persons had not received vaccine previously, one must seek elsewhere for the explanation. Perhaps the reaction can be classified as an anamnestic one. The reason for this response may lie in facts drawn from the investigations of Thomas and co-workers,[44]

who have demonstrated that serum from patients suffering from primary atypical pneumonia may give falsely positive reactions with other viral antigens which contain animal tissue.

The finding of negative complement-fixation tests does not necessarily rule out a virus disease, because as Hirst [45] points out, (1) some patients are poor reactors and their sera will always give less than a fourfold response; (2) certain persons with an initial high residual titer may fail to develop more antibody; (3) an occa-

TABLE 3.—*Results of Complement-Fixation Studies on Patients with a Clinical Diagnosis of Primary Atypical Pneumonia*

Patient	Date (1951)	Reciprocal of Highest Titer Showing Positive Complement-Fixation by Antigens: Influenza A	Influenza FM_1	Influenza B	Psittacosis Direct
F. C.	10/20	..	2	4	..
	10/30	2	2	8	..
	11/1	..	4	16	..
C. K.	10/19	..	2	8	..
	10/30	..	2	8	..
	11/9	..	4	32	..

TABLE 4.—*Clinical Symptoms and Tests Other Than Complement-Fixation on Patients with a Clinical Diagnosis of Primary Atypical Pneumonia*

Patient	Date (1951)	Cold Agglutination Test	X-Ray Finding	Clinical Symptoms
F. C.	10/24	Pos./1-32	Characteristic of primary atypical pneumonia	Characteristic of primary atypical pneumonia
	11/9	Pos./1-128		
C. K.	10/19	Pos./1-32	Characteristic of primary atypical pneumonia	Characteristic of primary atypical pneumonia
	11/2	Pos./1-128		

TABLE 5.—*Results of Repeated Complement-Fixation Tests on Two Specimens of Serum from Patient M. C.*

Date (1952)		Reciprocals of Highest Titer Showing Positive Complement-Fixation by Antigens							
Specimen	Test	Granuloma Venereum^M	Psittacosis^M	Influenza A^M	Influenza B^M	Granuloma Venereum^L	Psittacosis^L	Influenza A^L	Influenza B^L
4-2	4-2	..	32	2	4	..	..	..	..
4-2	4-16	..	..	±	4	..	..	..	..
4-16	4-16	..	..	4	4	..	..	..	..
4-16	4-18	..	..	4	4	..	32	8	4

sional patient may be infected with a virus of the group but his serum response is so strain-specific that it fails to react to the standard type antigen.

As viral and rickettsial antigens age, they tend to become anticomplementary. This tendency may not be detected at first in the anticomplementary control but may first be noted by the fact that on a given day all or nearly all the sera tested with the antigen give low positive titers. The antigen must then be discarded and replaced by a new supply.

False positive viral complement fixation reactions may be related to the phenomenon first reported by Maltener.[46] He has shown that many tissue suspensions which are used as antigens possess thromboplastic activity. Inactivated human

serum was found to enhance the thromboplastic activity of these antigens, and it was shown that a positive correlation existed between thromboplastic activity and false positive reactions.

Certain viral and rickettsial antigens consist of suspensions of infected chick embryo material. Occasionally egg protein-hypersensitive persons may react with these antigens to produce false positive reactions.[47]

Very occasionally the viral and rickettsial antigens now in use may give a positive reaction on one day and a negative reaction the next day with the same specimen of serum. The uncertainty of such a result can be overcome by employing at least two batches of a given antigen, preferably from different sources, with each specimen as suggested by Hammon.[48] Table 5 presents an example of this type of reaction obtained in our laboratory. The two specimens of serum employed in these tests were stored in flame-sealed ampules in the ice box.

Of 3,643 viral and rickettsial complement-fixation tests performed in our laboratory on the sera of hospital patients who were acutely ill at the time the first specimen was examined, 216 tests yielded results which subsequently were interpreted as giving positive confirmatory evidence of virus infection.[7] These results compare favorably with previous reports by Milzer [1] and Gauld.¶ They also compare very well with results of routine stool examinations for pathogenic enteric bacteria.[7]

Thus it can be seen that if some thought is given to both the performance and the interpretation of results obtained with properly executed complement-fixation tests for the virus and rickettsial diseases then these tests can be applied on a routine basis.

THE COMPLEMENT-FIXATION INHIBITION TEST

It has been known for a number of years that certain avian and mammalian sera containing antibacterial antibodies inhibit complement-fixation when they would be expected to give strongly positive reactions. For example, certain avian sera which give high agglutinin titers against Salmonella pullorum will not fix complement in the presence of S. pullorum antigen. These sera will, however, inhibit complement-fixation by rabbit antipullorum serum in proportion to the agglutinin titers of these avian sera. Wolf and co-workers [50] found a similar phenomenon in relation to Newcastle disease. In their work the sera of chickens infected with this virus failed to give results in direct complement-fixation tests. The same sera would, however, prevent fixation of the antigen by pig serum which had previously been shown to do so. Wolf applied these techniques to the development of an indirect complement-fixation test for this disease. Karrer, Meyer, and Eddie [51] applied this principle to the diagnosis of ornithosis in chickens and ducks when it was shown that the sera of these species did not give direct complement-fixation tests.

In our laboratory we have applied this method to the laboratory diagnosis of psittacosis-ornithosis and lymphogranuloma venereum in man.[52] We feel that where the clinical symptoms are typical of either disease and the direct complement-fixation test gives negative results the specimen should then be tested by this method. Serum which is to be studied in this way should not be stored in the deep-freeze but should

¶ Gauld, R. L.: Laboratory Diagnosis of Viral and Rickettsial Diseases, read before the 26th Annual Meeting of the American Society of Clinical Pathologists, Chicago, Oct. 27-30, 1947.

be kept in the icebox in chemically clean flame-sealed ampules. We have learned that this is necessary because deep-freeze storage may elicit prozones in these sera and the sera will then be inhibitory in the region of its prozone.[42]

In the inhibition test 0.2 ml. amounts of the patient's serum to be tested are added in descending twofold serial dilutions to 0.1 ml. of 1:4 dilution of antigen in a series of test tubes (Table 6). Two full units of complement are then added to each tube. These mixtures are next incubated at 37 C. for 30 minutes. One unit of homologous complement-fixing serum in 0.2 ml. volume (one unit is the highest

TABLE 6.—*Method of Testing Sera for Inhibitory Activity*

Patient's Serum (Serum W) 0.2 Ml.	Antigen 1:4 Dilution in Ml.	Complement 2 Units in Ml. *	One Unit of Positive Complement Fixing Serum (Serum H) in Ml. *	Sensitized Sheep RBC Suspension in Ml. †
1:2	0.1	0.2	0.2	0.4
1:4	0.1	0.2	0.2	0.4
1:8	0.1	0.2	0.2	0.4
1:16	0.1	0.2	0.2	0.4
1:32	0.1	0.2	0.2	0.4
1:64	0.1	0.2	0.2	0.4
1:128	0.1	0.2	0.2	0.4
1:256	0.1	0.2	0.2	0.4

* Incubate in 37 C. water-bath 30 minutes.
† Incubate in 37 C. water-bath until complement controls clear.

TABLE 7.—*Results of Complement-Fixation Test and Indirect Complement-Fixation Test on the Serum of Patient W.*

Patient's Serum in Dilutions of	Results of Direct Complement Fixation Test	Results of Indirect Complement Fixation
1:2	Negative	Negative
1:4	Negative	Negative
1:8	Negative	Negative
1:16	Negative	Negative
1:32	Negative	Negative
1:64	Negative	2+
1:128	Negative	4+
1:256	Negative	4+

dilution of serum giving 4+ fixation) is then pipetted into each tube of the series. The tests are incubated for a second period of 30 minutes at 37 C. A suspension of sensitized sheep red blood cells, in 0.4 ml. volume, is subsequently pipetted into each tube, and the tests are incubated for a third period at 37 C. until the complement controls [52] become clear.

The reading of the test can best be understood by consulting Tables 6 and 7. Since a known positive serum containing complement-fixing antibody is added to the test system (Serum H, Table 6), it would be expected that all the tubes would give 4+ positive results. Where the patient's serum contains the "inhibitory type of antibody," however, inhibition of complement fixation will occur in proportion to the amount of this substance present (Table 7), i. e., through the dilution of the patient's serum (Serum W, Table 6) where it is effective. Thus in the record of the direct test in Table 7 the result is negative throughout, whereas, in the

indirect test, inhibition of the activity of the standard complement-fixing serum proceeds through a dilution of 1 : 64. Inhibitive substance is present to this degree and may be regarded as evidence of infection. If a repeated test shows an increase (higher inhibition) in the activity of this inhibitive substance in a fourfold or greater degree, this can be interpreted as evidence of active infection.

SUMMARY

1. A review of the following laboratory tests which are employed in the laboratory diagnosis of viral and rickettsial diseases is presented: the neutralization test, the heterophile antibody test, the hemagglutination inhibition test, the cold hemagglutination test, the Streptococcus MG agglutination test, the complement-fixation test, and the complement-fixation inhibition test.

2. Each test is discussed from the following standpoints: (*a*) the principles involved in the method; (*b*) problems encountered in the performance of the test; (*c*) problems involved in the interpretation of the results obtained; (*d*) the applicability of the test to routine laboratory diagnosis of disease.

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THE TESTIS

IV. Idiopathic Eunuchoidism with Low FSH; Testicular Changes Secondary to Lesions in or Near the Pituitary and Secondary to Estrogen Therapy

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THIS PAPER is the fourth in a series devoted to a study of the normal and abnormal testis. Previous publications described the normal testis from fetal life to old age,[1] the abnormalities of spermatogenesis which were not associated with evidence of hormonal imbalance,[2] certain conditions believed to be primary in the testis that involved both the tubules and interstitial cells and were accompanied by hormonal imbalance,[3] and the testicular changes in precocious puberty.[4] The observations to be described here are the aberrations in the testis of a group of men with idiopathic or acquired hypopituitarism with respect to gonadotropins.

The clinical details of the cases incorporated in this work and the theoretical considerations of the abnormal hormonal patterns have been considered elsewhere,[5] and therefore only the briefest outline of these aspects will be discussed. The clinical and laboratory studies in the patients with idiopathic eunuchoidism and organic lesions of the pituitary included an evaluation of the endocrine status, estimation of the pituitary gonadotropins and 17-ketosteroids, and microscopic examination of testicular tissue. The same case numbers are retained throughout the published series. An additional 25 patients are included in the present study. These men were treated with estrogens for carcinoma of the prostate, and, after varying intervals, testicular biopsies or orchidectomies were performed.

IDIOPATHIC ENUCHOIDISM WITH LOW FSH

The hypogonadism of the 19 patients in this group, whose ages ranged from 16 to 48 years, resulted from pituitary hypofunction with respect to gonadotropins during puberty. In brief, these eunuchoid men had unduly long extremities, delayed epiphyseal closure, a small thyroid cartilage, and a high voice. There was a general decrease in body hair and no temporal recession. The phallus, testes, and prostate

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were small. Ejaculations were absent and erections variable; there was no gynecomastia. The laboratory data are indicated in Table 1, where it is evident that the urinary assays revealed an undetectible or low level of gonadotropins and low or low normal excretion of 17-ketosteroids. On the other hand, general body growth, basal metabolic rate, and insulin tolerance curves were within normal range, indicating the presence of other pituitary hormones. X-rays of the skull showed a normal sella turcica, and there was no clinical indication of an intracranial lesion.

All biopsy specimens were obtained before the institution of hormone therapy. The patients were grouped according to the stage of maturation attained by the testis at the time of biopsy.

The biopsy specimens of eight men (Cases 1 to 8) showed testicular tissue comparable to that of prepuberal children. The testicular cords were closely packed and

TABLE 1.—*Idiopathic Eunuchoidism with Low FSH*

Case No.	Age in Yr.	(Urinary) 17-Ketosteroids Mg./Day	(Urinary) * FSH M.U./Day
1	16	2.9	— **6**
2	18	2.0	— **6**
3	37	4.4	— **6**
4	20	9.8	— **6**
5	19	9.5	+ **3** — **6**
6	19	0.7	— **6**
7	21	?	— **6**
8	40	0.4	— **3**
9	20	4.0	— **6**
10	24	5.9	— **3**
11	20	2.6	— **3**
12	19	5.9	+ **6** —**13**
13	18	4.5	+**13** —**26**
14	21	3.5	+ **6** —**13**
15	47	9.8	— **6** + **6**
16	28	5.2	+ **3** — **6**
17	48	4.5	— **3**
18	35	4.7	— **6**
19	34	10.8	— **6**

* In both Table 1 and Table 2 the results of urinary FSH assays are condensed so that the highest level showing a positive response is preceded by a plus sign and the lowest level giving a negative response is preceded by a minus sign. If results were negative at all levels the figure shown is the lowest at which bioassay was performed. For the sake of brevity, tests at 6.5 M.U. are recorded as 6 M.U. The use of bold face indicates that the assay was done by the dialysis method, and the use of plain type, the nondialysis method. In these Tables a few FSH results, specially indicated, are reported in mouse units per 100 cc.

few of them had developed a lumen. The cords contained a granular syncytium in which were embedded several irregular layers of ovoid, deeply basophilic, homogeneous nuclei that lacked visible nucleoli. Large germ cells were present, which usually lay against the basement membrane, though many occupied a more central position (Fig. 1). Some of these cells were very large as if they were maturing into primary spermatocytes, occasional cells contained several nuclei, and usually a few mitotic figures could be found among them; some of the germ cells were disintegrating. The tunica propria was normal. The interstitial tissue was compact and closely knit with cells resembling fibrocytes and collagen fibers. A fine reticulum ran throughout both the interstitium and the outer layers of the tunica propria, but elastic fibrils had not formed. No Leydig cells were found.

Five patients (Cases 9 to 13) illustrated the first stages of tubular ripening (Fig. 2). The tubules had attained a somewhat greater diameter than that seen in

childhood or in the group described above. Small, ill-defined lumens were present occupied by degenerating cells and a granular precipitate. Further evidence of tubular development could be found in the partial metamorphosis of the indifferent cells of the tubule toward sustentacular cells of Sertoli. In the infantile tubule the

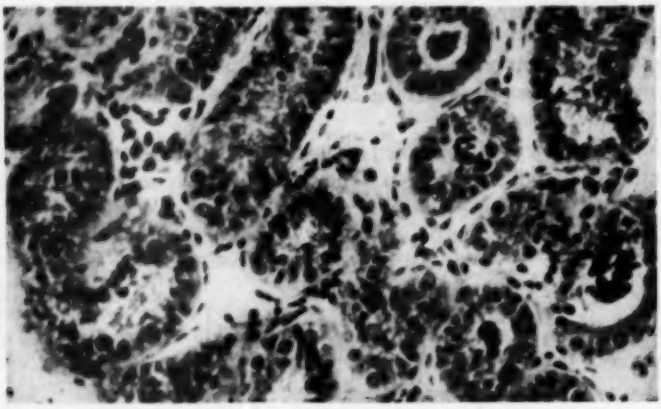

Fig. 1.—The immature testis of a 16-year-old eunuchoid patient. The small tubules are composed of undifferentiated cells punctuated by germ cells. The lumen of an occasional tubular segment contains disintegrating cells. The interstitial tissue is occupied by cells resembling fibrocytes. Phloxine-methylene blue stain; × 160.

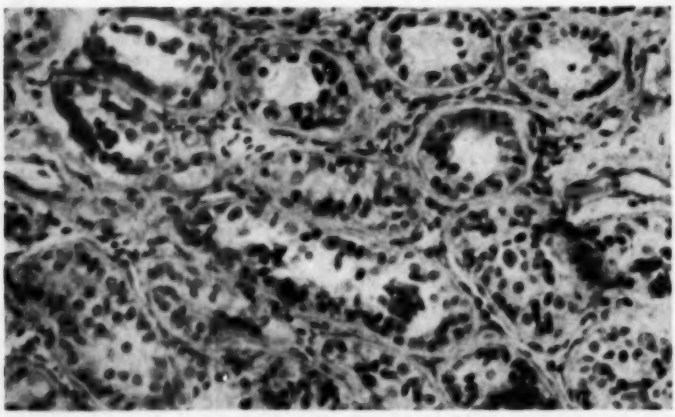

Fig. 2.—The testis of a 24-year-old eunuchoid patient showing slight maturation. The tubules have increased in diameter and more lumens have formed. There is slight thickening of the tunica propria. Many of the nuclei of the indifferent cells in the tubules stain less intensely than in the completely quiescent gland, and occasional nucleoli are visible. The interstitial cells are fibrocytoid. Phloxine-methylene blue stain; × 160.

nuclei of the undifferentiated syncytium are small and dense, while the cytoplasm is diffusely granular. Here, the nuclei were larger, and the granules of precipitated chromatin were distinct and widely dispersed. Nucleoli were visible, as in mature sus-

tentacular cells. In some cases, the ripening cells contained cytoplasmic crystalloids and fine vacuoles. Furthermore, the impression of cytoplasmic fibrillation imparted by fixation had become more distinct, as in the mature cell.

Germ cell activity was variable and independent of Sertoli cell maturation. Some germ cells seemed to be mature and had a relatively clear outer zone of cytoplasm with granules concentrated around the nucleus. In others, however, the cytoplasmic mass was slightly smaller and wholly granular. The latter cells may be transition forms between the indifferent cells of the tubule and mature germ cells—if such occurs. Cytoplasmic crystalloids were found occasionally in the germ cells when the tubules were relatively mature. Each testis in this group showed a slight to pronounced increase in collagen deposition in the tunica propria of the tubules (Fig. 3). The interstitial tissue contained no recognizable Leydig cells.

Six patients (Cases 14 to 19) showed more advanced tubular maturation but a concomitant increase in the severity and number of morphologic abnormalities.

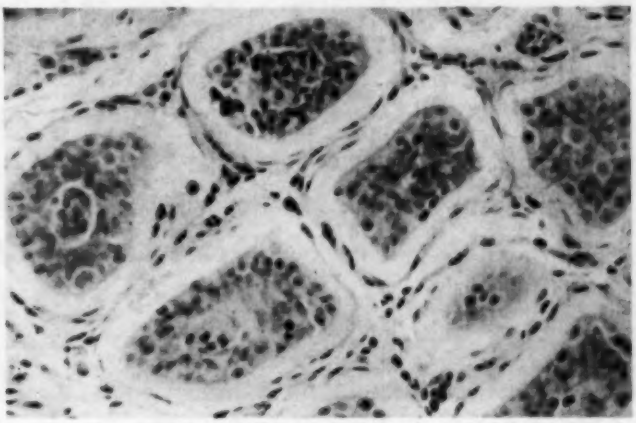

Fig. 3.—Immature testis of a 19-year-old eunuchoid patient illustrating the marked collagenous thickening of the tunica propria that may occur. Phloxine-methylene blue stain; × 160.

In two men (Cases 18 and 19) all the tubules had developed lumens and the sustentacular cells were readily recognizable, for the cytoplasm had acquired crystalloids and fine vacuoles and was distinctly "fibrillar." However, mitotic activity in the germ cells was at a low ebb. Here again there was collagenous thickening of the tunica propria and, in addition, definite multiplication of peritubular elastic fibrils. Leydig cells were not found in the interstitium.

In four patients (Cases 14 to 17) spermatogenesis was present, but the process was abnormal. The tubules were only slightly reduced in diameter from the normal and the Sertoli cells were mature. Spermatogenesis was spotty with premature desquamation of the cells into the lumens, where they were degenerating. The excessive dropping off of the gametogenic cells usually occurred at the primary spermatocyte stage (Fig. 4). A few sperm cells were present, but the majority were found in the tubular lumen and were not embedded in the Sertoli cytoplasm, which is their usual position in the normal testis. Many of the spermatogonia seemed to be "dormant" and were small with a densely staining, unbroken cytoplasm. Abnormal

collagen deposition thickened the tunica propria and several layers of elastic fibrils encircled the tubules. Occasionally spermatogenesis had ceased and the tubule was lined by atrophic Sertoli cells. Complete tubular sclerosis was not widespread.

In general, the loose interstitial tissue of these four biopsy specimens showed an increase in collagen. In one patient (Case 17) no Leydig cells were identified, but in the other three patients (Cases 14 to 16) a few of these cells had formed. They lay singly or in small groups and were small with an indistinct outline. A large proportion of them seemed to be disintegrating and a remarkable number contained cytoplasmic crystalloids. Usually the identification mark of these abnormal cells was the presence of crystalloids. The testis of one man showed, in addition to the above changes, Leydig cells that were four to five times the normal diameter, and the cytoplasm was partially or completely occupied by fine vacuoles. Many Reinke crystalloids were present in all the "foamy" cells.

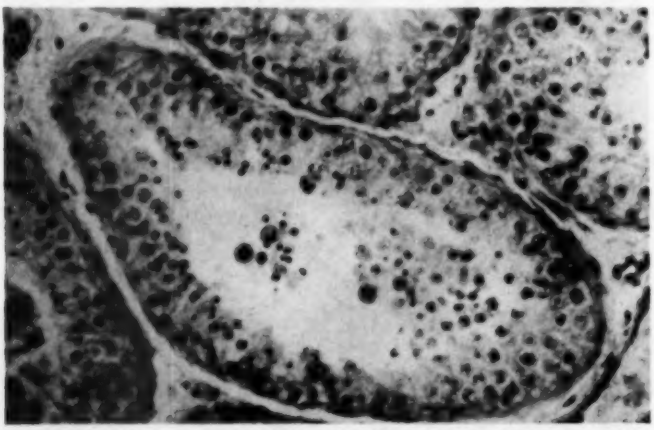

Fig. 4.—A tubule in the testis of a 48-year-old eunuchoid patient. The segment has reached almost adult dimensions, but spermatogenesis is not fully active. Generally the process is arrested at the primary spermatocyte level. Phloxine-methylene blue stain; × 160.

Comment.—From this study and the work of others it is apparent that patients with idiopathic eunuchoidism with low follicle-stimulating hormone (FSH) have immature testes, or they have partially mature testes that are abnormal.

The failure of testicular maturation appeared to result from a primary lack of gonadotropins. If the testis had never been stimulated it remained in the immature state for an indefinite period without showing structural abnormalities (Cases 1 to 8). Some patients did show enlargement of the tubules and partial maturation of the Sertoli cells but little spermatogenic activity and no development of Leydig cells (Cases 9 to 13). Here, however, the tubules were abnormal with thickening of the tunica propria. The testes of the remaining patients showed mature Sertoli cells and fairly active spermatogenesis but the gametic cells had desquamated in large numbers. The tunica was thickened and elastic fibrils had been deposited in its outer layers (Cases 14 to 19). The interstitial tissue of several of these patients contained abnormal Leydig cells. The finding of partial testicular maturation, abnormal spermatogenesis and faulty Leydig cells was interpreted to mean that there had been some

gonadotropic stimulation in the past which subsequently diminished, resulting in regressive changes in the gonad. Evidence for this hypothesis was found in the fact that four of the last six patients were married and apparently potent, and the men who had developed a demonstrable lesion in the pituitary in adult life showed the same testicular changes in the early stages of their disease as these patients with idiopathic eunuchoidism. Furthermore, if a eunuchoid patient has originally shown thickening of the tunica propria of the tubules, the administration of chorionic gonadotropin or testosterone will restore this layer to normal.[6] This finding is unexpected, as one usually considers collagen to be a peculiarly inert substance. If therapy is then discontinued, a thick tunica will reappear. The human testis is apparently incapable of returning to a normal immature state after it has been stimulated naturally or by gonadotropic therapy.

TABLE 2.—*Conditions Secondary to Organic Lesions in or Near the Pituitary*

Case No.	Age in Yr.	17-Ketosteroids (Urinary) Mg./Day	FSH (Urinary) * M.U./Day
20	19	2.1	— 3
21	28	2.4	— 6
22	23	8.7	— 3
23	26	4.0	— 6
24	74	2.2	— 3
25	72	1.3	—10/100 cc.
26	64	0.6	— 3
27	27	1.8	not done
28	56	1.8	not done
29	52	2.2	— 3
30	49	4.2	— 3
31	58	1.0	+ 6 & — 6
32	41	1.1	+ 3 — 6
38	18	0.8?	+ 3 — 6
39	46	3.2	+ 6 —13
40	35	3.3	+ 6 —26
41	55	3.5	+26 —32

* See footnote to Table 1.

TESTICULAR CHANGES SECONDARY TO LESIONS IN OR NEAR THE PITUITARY

Testicular abnormalities and hypogonadism follow the development of organic lesions in or near the pituitary gland. Seventeen men were studied, all of whom had hypogonadism and some of whom panhypopituitarism. The patients had either proved or presumptive evidence of damage to the pituitary. The diagnosis was confirmed at autopsy in five patients and by craniotomy and biopsy in four patients. In seven patients the symptoms, signs, laboratory findings, and skull roentgenograms indicated an intracranial lesion with pituitary gland involvement. One patient suffered from panhypopituitarism without roentgenologic evidence of an abnormal sella turcica or increased intracranial pressure. The proved lesions in and around the pituitary were the tumors, cysts, and injuries common to this area.

The pertinent laboratory data are shown in Table 2. The ages of the patients ranged from 18 to 74 years, while the approximate intervals between the first subjective symptom of disease and the study of the testicular tissue varied from 1 to 40 years.

The findings in the testis depended on several factors, namely, the age of the patient at the onset of his illness, the severity of pituitary damage, and the interval between the genesis of the pituitary lesion and the testicular biopsy.

Four patients (Cases 20 to 23) had developed an intracranial lesion before the completion of puberty and showed the various manifestations of eunuchoidism. The 17-ketosteroid values were low and pituitary gonadotropins were not detected in the urine.

The testis of the first patient (Case 20) showed no evidence of stimulation and was completely immature. The tubules were small and closely approximated, only occasional lumens were present and these contained desquamated cells. The cytoplasm of the undifferentiated syncytium was faintly "fibrillar" and the nuclei were small, densely packed with chromatin, and without visible nucleoli. A few germ

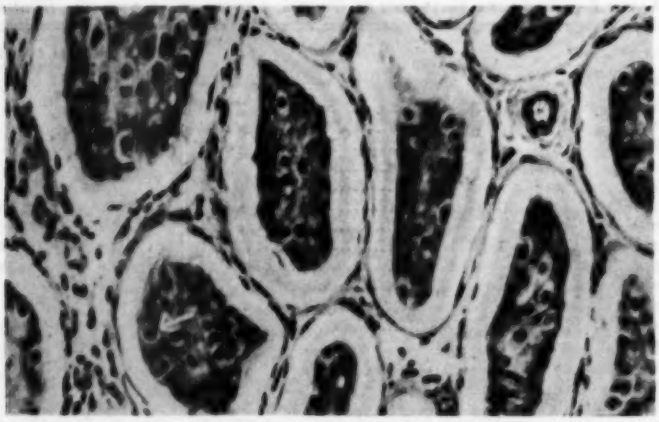

Fig. 5.—The testis of a 28-year-old man with a Rathke pouch cyst that had developed during puberty. The tubular cells are undifferentiated, though a few germ cells are present. There is marked collagenous thickening of the tunica propria. No Leydig cells have formed. Compare with Figure 3. Masson's trichrome stain; × 160.

cells were present, but mitotic activity was at a minimum. The tunica propria was slightly thickened. No Leydig cells had formed in the compressed, collagenous interstitial tissue.

The testes of the other three patients showed some signs of tubular maturation similar to the changes that take place in the normal gland at puberty. The tubules had attained a greater diameter than those of an inactive gland, and more lumens had formed. Importance was attached to the enlargement of the Sertoli cell nuclei as an indicator of tubular ripening. The fine chromatin granules in these nuclei were dispersed and the nucleoli were distinct. In these patients the cytoplasm of the maturing Sertoli cells was more obviously "fibrillar," and crystalloids and fine vacuoles were occasionally found. Unlike the normal testis, germ cell activity did not always keep pace with these indications of tubular maturation. The tubules were abnormal in other respects. There was collagenous thickening of the tunica propria, a deposition of peritubular elastic fibrils, and small groups of

tubules were completely sclerotic (Fig. 5). The interstitial tissue in each case showed an increase in collagen fibrils. Leydig cells were present in the testis of only one patient (Case 22). They were found in small groups, and individually the cells were small but could be identified by the crystalloids and pigment in the cytoplasm. Many of the cells seemed to be degenerating.

Cases 24 to 32 were men who had developed signs of an intracranial lesion with pituitary destruction after the years of puberty. The patients had an average age of over 50 years. Several of the married men had had children before symptoms occurred. The intracranial lesion had produced diplopia, failing vision, dizziness, headache, and vomiting. Evidence of pituitary damage was found in such symptoms and signs as a decrease in libido, potency, and hair growth, the occurrence of hot flashes, emotional changes, and mild or severe manifestations of hypothyroidism and adrenal insufficiency. The penis, testes, and prostate were either small or normal in size. The patients excreted undetectible or subnormal quantities of pituitary gonadotropins and the 17-ketosteroid excretion was far below normal.

These men illustrated the effect of withdrawing pituitary gonadotropins from the adult testis. By studying the group as a whole, one could arrange the histological changes in sequence. The abnormalities appeared to depend more on the degree of pituitary hypofunction than on the duration of hypofunction.

With routine staining techniques, the first testicular changes detected were a slight decrease in spermatogenic activity and excessive desquamation of the gametic cells, predominantly primary spermatocytes. No other tubular abnormality was found, and the tunica propria was not thickened. No obvious change had taken place in the Leydig cells, though possibly an excessive number of them were disintegrating or showed complete transformation of the cytoplasm into fine vacuoles. Following this stage, hypospermatogenesis became severer, and only a few dividing germ cells remained. Concomitantly fine lipoid droplets accumulated in the Sertoli cytoplasm. The tubules gradually decreased in diameter, while the number of collagen fibrils increased in the tunica propria and many elastic fibrils appeared in the peritubular zone (Fig. 6). In these testes the number of completely sclerotic tubules and the collagen content of the interstitial tissue were variable. An eosinophilic granular precipitate was sometimes scattered throughout the interstitium.

Subsequently, there was marked reduction in tubular diameter and the lumens were filled with Sertoli cytoplasm that had been converted into a foam of fine and coarse sudanophilic isotropic droplets (Fig. 7). The Sertoli nuclei were unchanged in appearance and formed an irregular line at the base of the cells. Varying numbers of primary germ cells were still present along the basement membrane and often they were multinucleated; there was no progression of spermatogenesis beyond this stage. The fibrillar tunica propria showed progressive thickening and was sprinkled with fine eosinophilic granules that did not have the tinctorial properties of collagen. Obvious multiplication had taken place in the peritubular lamellae, resulting primarily from a deposition of elastic fibrils (Fig. 8).

Whether the tubules showed severe hypospermatogenesis or more advanced atrophy, the abnormalities in the Leydig cells were remarkably consistent within this group of patients. The cells were reduced in number, coarse cytoplasmic granules were absent, and the number and distribution of vacuoles in the cytoplasm were

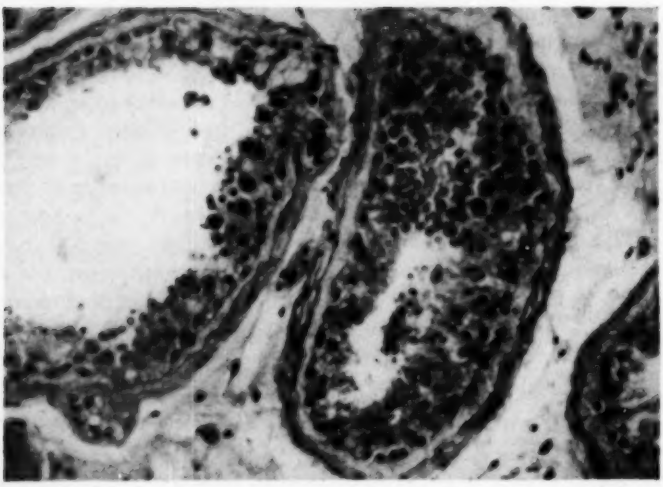

Fig. 6.—The testis of a 53-year-old patient with pituitary damage acquired after puberty. The tubules show a decrease in spermatogenic activity and thickening of the tunica propria contributed to by an increase in the number of elastic fibrils. Verhoeff's elastic tissue stain; × 160.

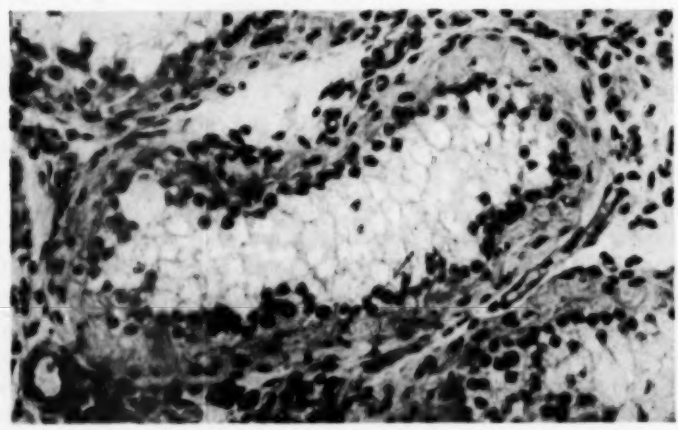

Fig. 7.—The testis of a 64-year-old man with pituitary injury acquired after puberty. The tubules are shrunken, only a few primary germ cells remain and the Sertoli cytoplasm is filled with sudanophilic isotropic globules. The tunica propria is thickened by collagen and elastic fibrils. The Leydig cells have disappeared and the interstitium contains an increased number of "fibrocytes." Phloxine-methylene blue stain; × 160.

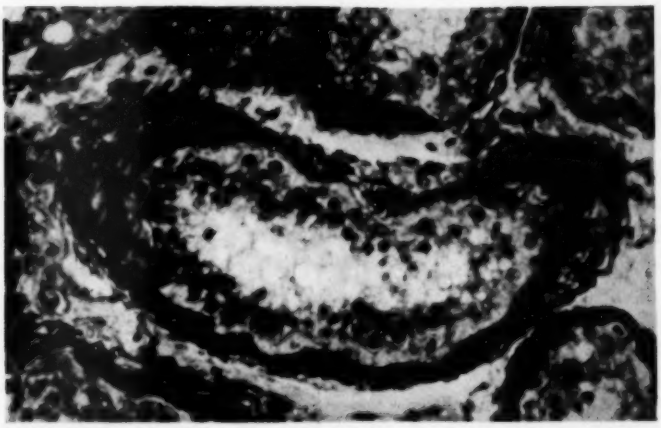

Fig. 8.—The testis of a 52-year-old patient with hypopituitarism that became manifest after puberty. The photograph illustrates the marked increase in elastic fibrils in the tunica propria. Verhoeff's elastic tissue stain; × 160.

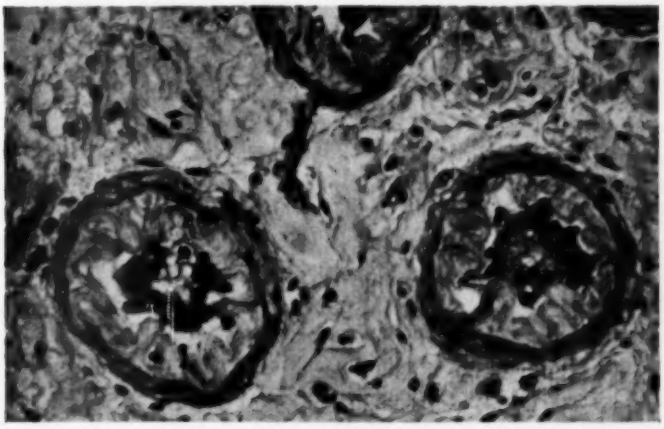

Fig. 9.—Testicular tubules in a 22-year-old patient with pituitary damage. The lumen is occupied by Sertoli cells in which a few cytoplasmic, sudanophilic droplets remain; there are no germ cells. The tunica propria shows a marked collagenous thickening; the outer laminae were composed predominantly of elastic fibrils. The interstitial tissue is highly collagenous and Leydig cells have disappeared. Phloxine-methylene blue stain; × 160.

abnormal. Often the cytoplasm was filled with fine vacuoles. Anuclear, and at times wholly vacuolated, cytoplasmic smudges were numerous, as well as cells showing nuclear pyknosis. The pigment granules in the Leydig cells were often unusually abundant; the formation of crystalloids was variable. The collagen content of the interstitial tissue was inconsistent. The adventitia of the arterioles was thickened.

In the final stage of testicular failure, complete tubular sclerosis had occurred (Fig. 9). The lumen of the tubules was obliterated by the fibrillar tunica propria, the basement membrane had disappeared, and each tubule was distinctly outlined by multilayered peritubular elastic lamina. The interstitial tissue at this time was highly collagenous and devoid of Leydig cells.

Cases 38 to 41 have been treated separately in this study because the testicular abnormalities differed in certain respects from those of the foregoing patients. All four patients had roentgenologic evidence of pituitary or juxtasellar lesions. In Table

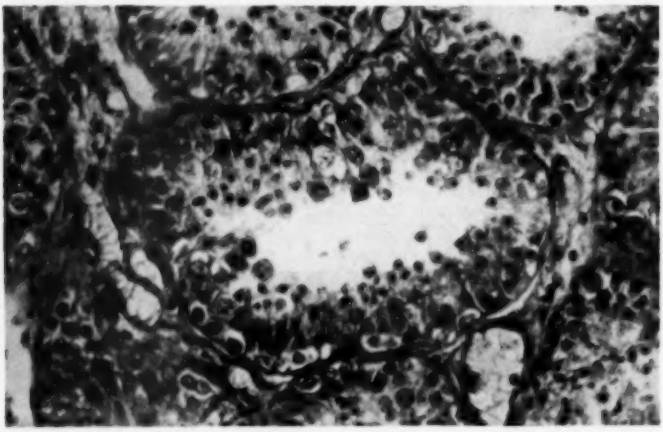

Fig. 10.—Cross section of a testicular tubule in an 18-year-old patient with pituitary damage. There is moderate hypospermatogenesis. Leydig cells were not found in the interstitial tissue. Phloxine-methylene blue stain; × 160.

2 it is shown that the four men were in different decades of life. Their excretion of urinary 17-ketosteroids was distinctly low, but the excretion of pituitary gonadotropins was normal. The interstitial tissue of none of these patients contained visible Leydig cells, but a varying degree of spermatogenic activity still existed. In Cases 39 to 41 the testes showed hypospermatogenesis with excessive desquamation of gametic cells and increased collagen content in the interstitial tissue (Fig. 10). The testis in Case 41 showed, in addition, generalized thickening of the tunica propria and many of the tubules were completely sclerotic. Case 38 was a young man 18 years old who had not developed secondary sex characters at puberty. Pituitary gonadotropins were just detectible in the urine and the 17-ketosteroid excretion was low. The testicular picture in this case was one of partial maturation without regressive phenomena. The sustentacular cells of the tubules were mature, but spermatogenesis was underactive though a few sperm cells had formed.

TESTICULAR CHANGES SECONDARY TO ESTROGEN THERAPY

The testes of 25 men who had been given estrogen for varying intervals before the performance of a testicular biopsy or orchidectomy were studied. Twenty-two of the patients had carcinoma of the prostate and two were acromegalic.* The last patient was a precocious boy 7 years of age, who was given small doses of estrogen in the hope of precluding misadventures in his neighborhood. Biopsy specimens were taken from only three patients before and after treatment, and, therefore, uncertainty exists concerning the activity of the testes before therapy.

In general, men receiving like quantities of estrogen over the same period showed similar testicular changes. Obviously, irregularities in the progression of testicular failure would be produced by such factors as the intensity of the estrogen treatment, the route by which it was given, and, theoretically, the sensitivity of the pituitary gland and testis.

In brief, the histological changes produced in the testis by the administration of estrogens were similar to those found in men with organic lesions in the pituitary.

In the following paragraphs a brief description will be given of the changes in the testis after increasing time intervals from the beginnings of diethylstilbestrol (stilbestrol) therapy. The dose per day differed from patient to patient and varied from 1 to 10 mg.

After the administration of approximately 45 mg. of diethylstilbestrol during a five-day period, the tubules showed a definite drop in spermatogenic activity following a period of irregular, but apparently excessive, desquamation of the gametic cells into the lumen (Fig. 11). This was accompanied by an accumulation of lipoid vacuoles in the Sertoli cytoplasm. These abnormalities preceded, but were followed closely by, an increase in the collagen content of the tunica propria. In some glands, at this time, very few Leydig cells could be found, and they were small, finely granular, and contained many pigment granules. In other testes, the Leydig cells were present in normal numbers, but an abnormal number were disintegrating.

At the end of a week with an approximate total dose of 70 mg. of diethylstilbestrol there was severe hypospermatogenesis with very few sperm cells, further accumulation of Sertoli lipoid, and an increase in thickness of the tunica propria.

In two weeks with 160 mg. of diethylstilbestrol only a few spermatogonia and spermatocytes remained and no sperm cells had formed. The Sertoli lipoid and tunica propria thickening had further increased. At this point, there was focal augmentation of peritubular elastic fibrils. The few small Leydig cells that remained contained a great deal of pigment and often many fine vacuoles or a large hyaline droplet.

With larger doses of diethylstilbestrol over a longer period of time there was some variation in the severity of the testicular damage. The usual abnormalities were similar to those found in severe mechanical injury of the pituitary (Cases 24 to 32). The shrunken tubules were lined by Sertoli cells containing a foam of isotropic lipoid droplets (Fig. 12). The tunica propria was greatly thickened, and elastic fibrils surrounded the tubules. Of the spermatogenic cells only the spermatogonia remained. Indeed, spermatogonia and recognizable Leydig cells persisted for

* Dr. G. G. Smith permitted publication of clinical details of the majority of the patients with cancer of the prostate.

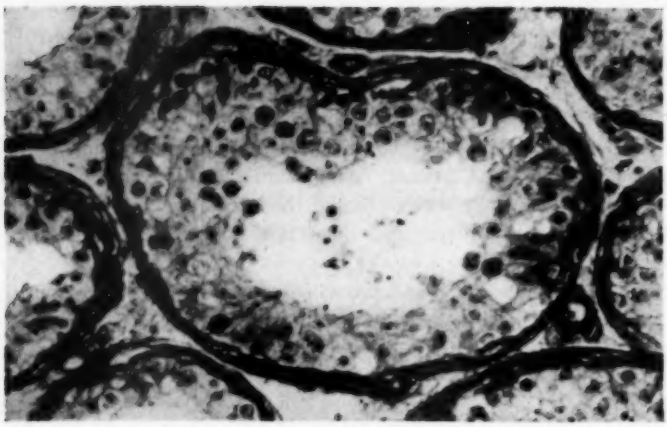

Fig. 11.—A testicular tubule of a patient given 43 mg. of diethylstilbestrol for a period of five days for carcinoma of the prostate. There is severe hypospermatogenesis and slight thickening of the tunica propria. Phloxine-methylene blue stain; × 160.

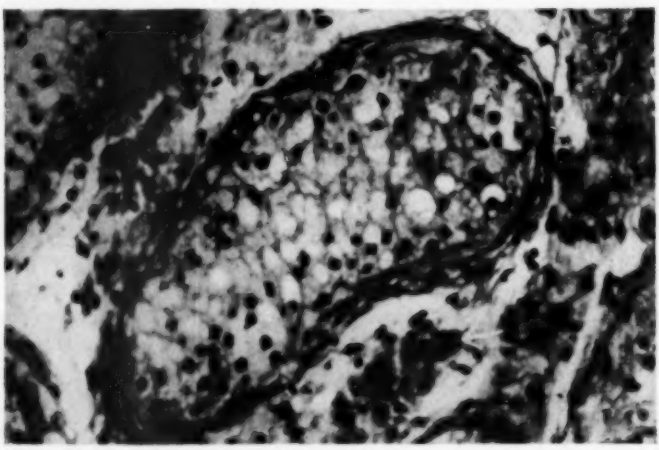

Fig. 12.—A testicular tubule from a patient with carcinoma of the prostate who was given diethylstilbestrol for six months. He had been off treatment for six weeks. Spermatogenesis has ceased, though a few primary germ cells remain. The tunica propria is thickened by collagen and elastic fibrils. Leydig cells (arrow) in small groups are present (or have reappeared). Phloxine-methylene blue stain; × 160.

a long time. For example, the testes of a patient given a total dose of 360 mg. of diethylstilbestrol during a six-month period still retained a few germ cells, and occasional Leydig cells remained in the interstitial tissue. The latter were distinctly abnormal, being small, highly vacuolated, and often disintegrating; usually they contained pigment and sometimes crystalloids. In a man given 575 mg. of diethylstilbestrol during a 250-day period, germ cells had disappeared, but a few abnormal Leydig cells remained. With larger total doses over greater intervals the Leydig cells disappeared.

Beyond this point, the tubules gradually lost the sudanophilic droplets and the tunica propria filled in the lumen. The end-stage was seen in testes that consisted of small sclerotic tubules separated by dense bands of collagen.

Comment.—The patients with pituitary injury could be divided into distinct categories with respect to the findings in the testis. With destruction of the pituitary before puberty or the initiation of gonadotropic activity, the testis remained in an immature state without evidence of inherent abnormalities. Destruction of the pituitary after puberty, when the testis had matured, resulted in a pattern of testicular change that was constant and apparently distinctive of gonadotropin suppression. The first obvious abnormality was a subsiding spermatogenic activity accompanied by excessive desquamation of gametogenic cells. As spermatogenic activity decreased, isotropic lipoid droplets became more numerous in the Sertoli cytoplasm. Concomitantly, collagen deposition occurred within the tunica propria, and elastic fibrils formed in the outer layers of the tunica. In the final stages of tubular atrophy the lumen was obliterated by collagen fibers and the basement membrane disappeared so that the tubules were converted into a fibrous cord. The tubular changes were accompanied by distinct abnormalities in the Leydig cells culminating in atrophy when they were no longer recognizable. The abnormalities in these testes appeared to depend primarily on the degree of pituitary hypofunction rather than the duration of hypofunction.

Cases 38 to 41 were of particular interest as the testes of these patients showed spermatogenesis without recognizable Leydig cells in the interstitial tissue. The urinary excretion of gonadotropins in these men was normal, but the 17-ketosteroids were low. On the surface, this finding would imply that spermatogenic activity is independent of Leydig cell function. However, the biopsy specimens were examined after routine staining methods and the appearance of the interstitial tissue is deceptive under such conditions. For example, at the beginning of puberty, tubular activity is apparent before Leydig cells are recognizable. Yet fat stains will demonstrate sudanophilic droplets in cells resembling fibroblasts.† Therefore one hesitates to draw conclusions on the activity or inactivity of the interstitial tissue before more exact methods have been developed. Nonetheless, a comparison can be made between these four men and four of the patients with iodiopathic eunuchoidism (Cases 14 to 17) whose testes showed spermatogenic activity despite the fact that the Leydig cells were either distinctly abnormal or absent. This finding has been discussed in a previous publication [4] and by Segaloff,[9] with the interpretation that there was lack of luteinizing hormone (LH) secretion in the presence of normal FSH secretion. Recently McCullagh, Beck and Schaffenburg [10] and Landau [11] have

† References 7 and 8.

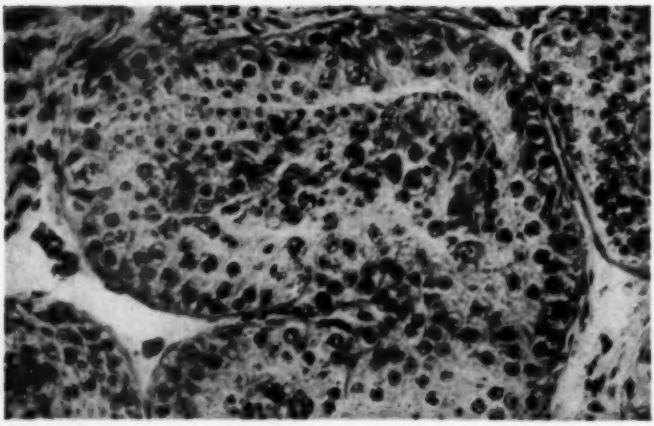

Fig. 13.—The testis of a 7-year-old boy with central nervous system precocity. There is active spermatogenesis. Mallory's phosphotungstic acid hematoxylin stain; × 160.

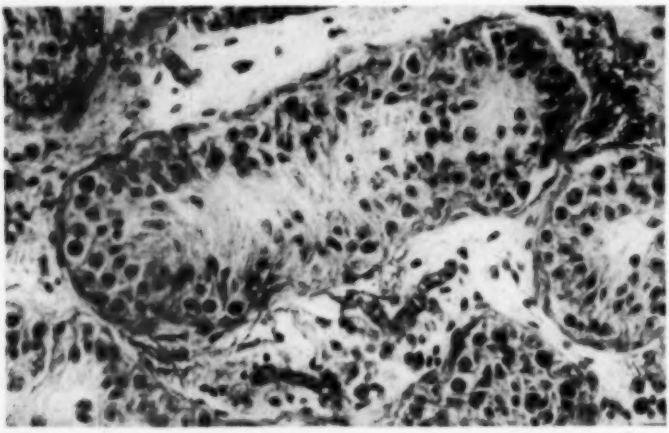

Fig. 14.—The testis of the patient illustrated in Figure 13 after 1 mg. of diethylstilbestrol daily for one month. Spermatogenesis is severely depressed, and only a few spermatogonia and primary spermatocytes remain. The lumens of the shrunken tubules are filled in with Sertoli cell cytoplasm. Mallory's phosphotungstic acid hematoxylin stain; × 160.

presented cases of hypogonadism with spermatogenesis and evidence of decreased androgen production. The Leydig cells of these patients were either absent or diminished in number.

The testicular atrophy that occurs during estrogen therapy is presumed to result from suppression of pituitary gonadotropin production. Indeed the morphological changes that occurred in the testes during estrogen therapy and after mechanical injury to the pituitary were strikingly similar.

It would be interesting to know how much estrogen is necessary to produce tubular sclerosis and thereby an effective castration. It is known that a small amount of estrogen will suppress testicular activity quite impressively.[12] For example, the testis of a precocious child 7 years of age showed active spermatogenesis and Leydig cells (Fig. 13). At the age of 9 he was given 1 mg. of diethylstilbestrol daily for one month, when another testicular biopsy was performed. Microsections showed a reduction in tubular size, absence of sperm and spermatids, and excessive desquamation of spermatogenic cells (Fig. 14). No morphologically distinct Leydig cells were visible. Judging from this small series it seemed that about 160 mg. of diethylstilbestrol given over a period of two weeks was sufficient to produce tubular failure to a point where only a few germ cells remained, great thickening of the tunica propria, and obvious degenerative changes in the few recognizable Leydig cells.

A testis of a 77-year-old man with cancer of the prostate was subjected to biopsy before treatment with estrogens. The gland showed active spermatogenesis and normal Leydig cells. Over the next 67 days the patient received 181 mg. of diethylstilbestrol, and at this point another testicular biopsy was performed. At this time, the tubules were shrunken and the lumina obliterated with Sertoli cytoplasm that was filled with sudanophilic vacuoles. Spermatogenesis had stopped, and only a few spermatogonia remained. The tunica propria was thickened, and the elastic fibrils around the tubules had multiplied. The Leydig cells had disappeared.

Another point of some practical interest concerns the reversibility of these testicular changes. Can the gland return to normal in the face of profound histologic abnormalities? This question cannot be answered at the moment, but possibly a clue is offered by a patient 70 years of age who was instructed to take, and presumably ingested, 350 mg. of diethylstilbestrol over a period of 76 days. From the foregoing, one would assume that his testicles at the conclusion of the therapy would contain only a few primary germ cells and show marked thickening of the basement membrane and, perhaps, no Leydig cells. After this treatment an interval of four months elapsed before orchidectomy was performed. The testes showed all conditions of tubules from normal to complete sclerosis and the Leydig cells appeared to be normal. With this dosage of estrogen it seemed fair to assume that the interstitial cells had disappeared at the completion of therapy. If this were so, the Leydig cells must have reappeared after the drug was discontinued.

SUMMARY

The testes of men with hypogonadotrophic eunuchoidism showed varying degrees of tubular immaturity often complicated by fibrillar thickening of the basement membrane. Spermatogenesis, when present, was abnormal. Generally Leydig cells were not recognizable, though occasionally abnormal forms were encountered.

The testes of men with postpuberal pressure atrophy of the pituitary showed progressive atrophic change that followed a definite pattern culminating in complete tubular sclerosis and atrophy of the Leydig cells. The severity of the change appeared to depend more on the degree than on the duration of hypopituitarism. If hypopituitarism had developed before puberty the testes remained in the immature state. No difference was noticed between the immature testes of idiopathic eunuchoidism and prepuberal hypopituitarism resulting from pituitary injury.

The testes of patients given estrogen therapy showed atrophy similar to that found in men with direct mechanical injury to the pituitary. Relatively small amounts of estrogen produced profound, and perhaps irreparable, changes in the morphology of the testes.

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RELATION OF AGE TO THE HORMONAL CONTENT OF THE HUMAN ANTERIOR HYPOPHYSIS

Effect of Various Physical and Chemical Agents on Thyrotropic, Corticotropic, and Parathyrotropic Hormones

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THE EVALUATION of the state of hypophyseal activity on morphological grounds remains difficult despite attempts at correlating specific functions of this gland with increases or decreases in the relative numbers of certain cell types. As Carlson [1] points out, except for those instances of sizeable pathological lesions, the gland appears, anatomically and cytologically, remarkably stable between the ages of 20 and 80. There are many diseases of varying degrees of severity and of varying duration which point to some metabolic defect involving hypophyseal function, without supporting anatomical evidence on postmortem examination.

It, therefore, appears important that a method be developed for estimating the hypophyseal content of various tropic hormones in a single gland for adequate clinical-pathological correlation in various metabolic diseases involving pituitary function. However, it should be pointed out that even such information would not permit a complete correlation, since it would be necessary to know not only the pituitary content of various tropic hormones but also the ability of the gland to deliver the latter into the blood stream as indicated by blood hormonal levels and the ability of the target organ to respond to the hormonal stimulus. At the present time, not even the initial step in this cycle has been accomplished. The present report, therefore, represents an attempt to develop a technique for estimating thyrotropic, corticotropic, and possible parathyrotropic content of the human hypophysis.

In these investigations I have adopted the technique of subcutaneous implantation which Loeb and co-workers* have utilized in studying the hypophyses of various animal species and which Saxton and Loeb [7] have used in studying the thyrotropic and gonadotropic hormones of the human pituitary. The present study is, in part, therefore, an extension of the observations of Saxton and Loeb; in fact, some of the specimens utilized in this study were made available by the latter investigators.†

As a starting point, this report deals specifically with possible age variations in thyrotropic, corticotropic, and parathyrotropic content of the human hypophysis. The guinea pig has been selected as the test animal since I have previously reported

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From the Department of Pathology, Division of Laboratories, of the Jewish Hospital, and the Department of Pathology, St. Louis University School of Medicine.

* References 2 to 6.

† Dr. Leo Loeb gave advice and guidance in these investigations.

on age changes in the thyroid, adrenal, and parathyroid glands of a large series of guinea pigs,‡ and certain of the latter data have served as control material in the present study.

MATERIAL AND METHODS

One hundred fourteen human hypophyses were implanted into an equal number of female guinea pigs weighing between 150 and 200 gm. In most instances, the gland was quartered and one-fourth implanted subcutaneously daily for four days; the animals were then autopsied on the fifth day. In some instances, smaller fragments were implanted over a longer period of

TABLE 1.—*Primary Cause of Death*

Pathological Diagnosis	Cases, No.
Accidental and traumatic	51
Sequelae of arteriosclerosis	22
Malignant disease	12
Postpartum complications and postabortion septicemia	11
Pneumonia	3
Cardiac syphilis	3
Hydrocephalus	3
Brain abscess	2
Pulmonary tuberculosis	2
Sequelae of infant diarrhea	1
Osteomyelitis and septicemia	1
Chronic emphysema	1
Epilepsy	1
Meningococcus meningitis	1
Total	114

TABLE 2.—*Age Distribution of Human Hypophyses*

Age Group, Yr.	Hypophyses, No.
0-10	11
11-20	5
21-30	22
31-40	21
41-50	15
51-60	29
61-70	11
71 and older	9
Total	114

time. The method of division and the period of implantation appeared to have no significant influence on the level of mitotic activity and all results have therefore been combined. Neither the sex of the donor nor the disease to which the patient finally succumbed appeared to influence, to any notable degree, the hormone content of the hypophysis. The primary cause of death in the 40 females and 74 males whose pituitaries comprise this series is tabulated in Table 1.

Varying degrees of postmortem autolysis probably accounts, in large part, for the relatively wide range of variation in results, but this appears to be fairly evenly distributed through all age groups. The number of hypophyses tested in each age group is shown in Table 2.

In some experiments an attempt was made to determine whether or not pretreatment would yield a content of a specific tropic hormone greater than apparent in untreated fresh glands.

‡ References 8 to 11.

Accordingly, some of the pituitaries included in this age series were immersed in chemical solutions or exposed to various degrees of heat or cold for varying periods of time before implantation; the type of treatment is noted in the appropriate section. The measurement of hormonal content was derived from a comparison of mitotic counts in test animals with those obtained from normal guinea pigs of the same age group; as stated, the latter data were obtained from material previously reported.[10] A comparison was also made between the results in test animals receiv-

TABLE 3.—*Effect of Physical and Chemical Agents on the Thyrotropic, Corticotropic, and Parathyrotropic Content of the Human Anterior Hypophysis*

Agent	Duration of Exposure	Number of Guinea Pigs	Hormone Content *		
			Thyrotropic	Corticotropic	Parathyrotropic
Cold					
0 C	24 hr.	6	+	+	—
Heat					
50 C	15-90 min.	8	+	+	—
56 C	15-90 min.	12	+	+	—
Normal saline	24 hr.	4	+	+	—
Sodium benzoate					
1-2%	72 hr.	6	+	+	—
Ethyl-alcohol					
50%	24 hr.	2	+	+	—
70%	24 hr.	2	+	+	—
95%	1-7 days	6	+	+	—
95%	28 days	2	—	—	—
Acetone	24 hr.	3	+	+	—
Ether	7 days	4	+	+	—
Sodium lactate †	24 hr.	2	+	+	—
Sodium butyrate †	24 hr.	2	+	+	—
Sodium oleate †	24 hr.	3	+	+	—
Sodium sulfate					
6%	72 hr.	14	+	+	—
12%	72 hr.	3	+	+	—
½ Saturated	72 hr.	2	+	+	—
Saturated	72 hr.	2	+	+	—
50 % Ethyl alcohol & sodium sulfate					
6%	7 days	2	+	+	—
12%	7 days	2	+	+	—
Saturated	7 days	2	+	+	—
Formalin					
0.25%	7 days	2	+	+	—
0.50%	7 days	16	+	+	—
1.0%	7 days	5	—	+	—
1.5%	7 days	2	—	+	—
2.0%	7 days	2	—	+	—
1.5% Formalin and 95% ethyl alcohol	7 days	2	—	+	—

* + indicates tropic activity; — indicates absence of tropic activity.
† 1 M solution.

ing treated pituitaries and those obtained from guinea pigs implanted with untreated human hypophyses of the same age group. When mitotic counts were higher than in controls and within the range of variation obtained from animals receiving untreated glands, it was concluded that the chemical or physical exposure was without significant effect; in such instances the results were included in the appropriate age group in order to present a sufficiently large series to be significant. This is also the basis on which results are tabulated in Table 3, which deals specifically with chemical and physical exposures.

Mitotic activity in the thyroid and parathyroid glands, as well as in the adrenal cortex, was determined according to methods previously described.[10] Mitotic counts of the thyroid were recorded as the average number of mitoses per gland (both lobes), and those of the parathyroid

glands as the number of mitoses per 10,000 cells. With regard to the latter glands, the average number of epithelial cells per unit field was also determined, since this figure indicates changes in the average cell size; however, the latter data are not included since they did not vary significantly from those obtained in normal guinea pigs of the same age group. Mitotic activity in the adrenal cortex was expressed as the average number of mitoses per section.

RESULTS

The Effect of Various Physical and Chemical Agents on the Thyrotropic, Corticotropic, and Parathyrotropic Content of the Human Anterior Pituitary.—The experiments illustrated in Table 3 represent an attempt to separate hormonal effects by physical and chemical agents similar to those used by Loeb and co-workers § with

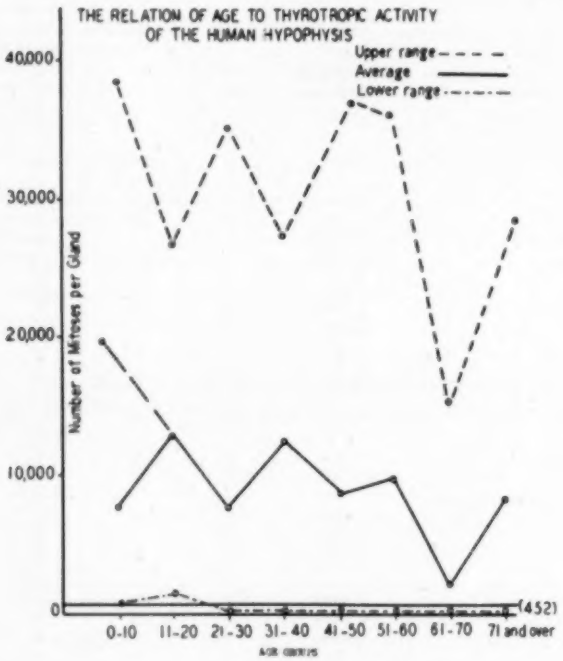

Chart 1

hypophyses of various animal species. It is obvious from these data that we have been unable to demonstrate any parathyrotropic activity in the human hypophysis by these procedures. On the other hand, the thyrotropic and corticotropic hormones appear to be resistant to many of these agents. The effective concentration of these endocrine substances in the human hypophysis is apparently unaltered by freezing at 0 C. for 24 hours, or by exposure to a temperature of 50 or 56 C. for as long as 90 minutes. Since normal saline or a preservative, sodium benzoate, were particularly considered as possible media for storing pituitaries for short periods, the glands were immersed in these solutions at room temperature for the periods shown in Table 3; no appreciable change was detected in the effectiveness of subsequent

§ References 2 to 7.

subcutaneous implants. Thyrotropic and corticotropic hormones also appeared to be resistant to common fat solvents, such as ethyl alcohol and acetone and ether, except after immersion for very long periods (95% ethyl alcohol for 28 days). Thyrotropic and corticotropic activity also appeared to be unchanged by immersion in 0.1 M solutions of the sodium esters of lactic, butyric, and oleic acids for 24 hours. Further, these hormones were remarkably resistant to even very concentrated solutions of sodium sulfate and to a combination of this compound and 50% ethyl alcohol. A most interesting effect was obtained with varying dilutions of formalin, which in concentrations of 1.0% or greater inactivated thyrotropic hormone, but had no appreciable effect on corticotropin; this differential effect was not altered by a mixture of 1.5% formalin and 95% alcohol. In general, pretreatment which

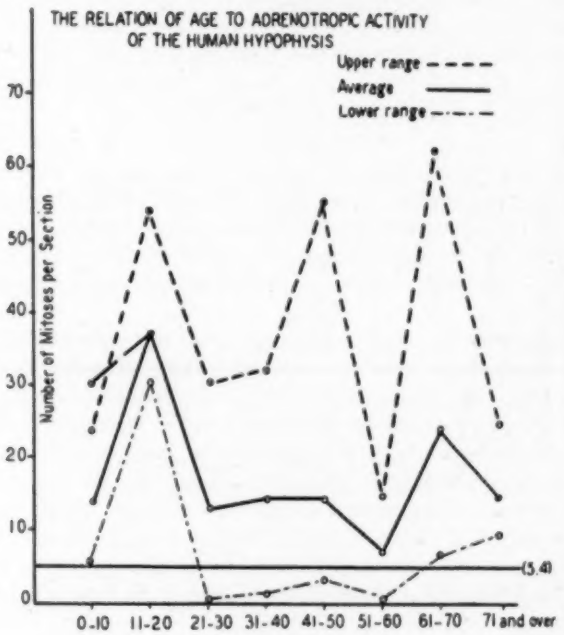

Chart 2

did not destroy tropic activity also did not give results indicating a higher content of tropic hormone than apparent in untreated glands.

The Relation of Age to Thyrotropic, Corticotropic, and Parathyrotropic Activity of the Human Hypophysis.—The influence of age on the thyrotropic content of the human anterior hypophysis is shown in Chart 1. The thyroid glands of untreated guinea pigs of the same age group and sex (female) showed an average count of 452 mitoses per gland, and this is indicated by the straight line near the abscissa of the graph. The range of variation for such normal females lies between 120 and 960 mitoses per gland. There was, therefore, a significant thyrotropic stimulation in all age groups. The spaced line leading from the age group 0 to 10 years to the point representing the average mitotic count for the next age group represents an average,

corrected for pituitary weight, in the youngest age group. If such correction is valid, there appears to be a higher thyrotropin content per unit weight of pituitary in the youngest age group than in succeeding decades. After this, there is a fairly constant thyrotropin content in all groups, with the exception of the decade from 61 to 70 years, in which a low average mitotic count was obtained. The reason for this is not obvious, although it may have been due to a greater number of hypophyses showing postmortem degeneration; it apparently represents some extraneous factor, since older hypophyses produce essentially the same average mitotic counts as between ages of 41 and 60 years. As noted previously, there was considerable variation in mitotic counts in each age group, but there was no distinct lack of high mitotic

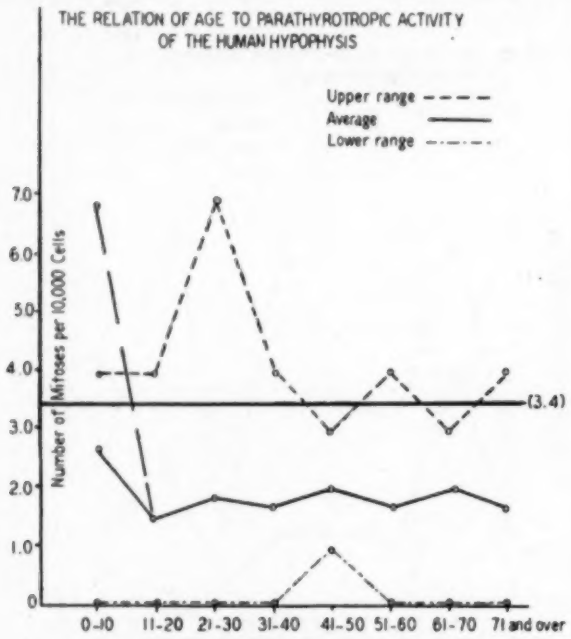

Chart 3

counts in animals receiving the pituitaries of old persons and low counts were occasionally obtained from all age groups.

The results of tests for corticotropic hormone content were comparable to those for thyrotropic hormone (Chart 2). The adrenal glands of untreated female guinea pigs of the same age group showed an average of 5.4 mitoses per section, with a range of variation between 1.0 and 8.2. Again, the spaced line leading from the youngest group to the next age group represents a possible correction for pituitary weight in young persons; the greatest corticotropic content per unit mass of tissue appears to be present in the first two age periods. In general, the average for the experimental group was distinctly above the control level except the group 51 to 60 years of age, but there was no real diminution with age, since succeeding periods were well above control levels and exhibited about the same degree of activity as adult glands below 50 years of age. In fact, the highest average mitotic count occurred in

the adrenal cortex of a guinea pig which received implants of hypophysis from a 68-year-old man. Low mitotic counts in test animals are probably explainable on the basis already mentioned.

In the experiments dealing with the effects of physical and chemical agents we failed to observe evidence of parathyrotropic activity. While some of the latter data are included in the results illustrated in Chart 3, omission of such data would not appreciably alter the curve of parathyrotropic content as related to age. With the exception of the youngest age group, which shows a very high average mitotic count when correction is made for pituitary weight, and a few scattered high counts in other age groups, mitotic activity was consistently lower in experimental animals than in control female guinea pigs. The average mitotic activity in the parathyroid glands of guinea pigs receiving human anterior pituitary was at a remarkably constant low level in all age groups. As stated previously, data is not included on average parathyroid cell size because there was no significant deviation from that obtained in control animals.

COMMENT

The present observations permit at least the conclusion that the guinea pig thyroid and adrenal glands respond readily to stimulation by appropriate human tropic hormone, and that this species is, therefore, a suitable test animal. The determination of an age curve is a necessary initial step for the purpose of providing a baseline to test the sensitivity of pituitary implantation into the guinea pig as a method for estimating hypophyseal content of tropic hormones in various metabolic disease states.

Although the technique utilized in the present report can be expected to provide only an estimate of specific tropic hormone content of the implanted hypophysis, the failure to find a diminution of such hormones with age does not necessarily indicate a lack of sensitivity of the method, nor the limit of response of the glands of the test animal. With regard to the latter, I have observed greater responses in both the thyroid and adrenal glands of the guinea pig to implantation of a variety of animal pituitaries.‖ The failure to observe a progressive loss of tropic hormone with advancing age should be evaluated in relation to the following review of morphological and physiological information relevant to this problem.

According to Saphir,[12] the average weight of the hypophysis between the ages of 10 and 20 is 0.56 gm. Parsons [13] has observed that the gross weight and size of the human pituitary changes little between the ages of 20 and 80; the average for both sexes is from 0.60 to 0.75 gm. In the latter series, there is a slight decline in pituitary weight in women after 60 and a slight increase in men past 40. In a very large series of 800 hypophyses, Simmonds [14] observed only a slight decrease in pituitary weight of both men and women after the age of 40.

With regard to cytological studies on the hypophysis, there appears to be general agreement that with increasing age there is a progressive decrease in the relative numbers of eosinophiles and an increase in chromophobes; this obtains in humans as well as in rats and chickens.¶ Mitoses are abundant in the eosinophiles and chromophobes in immature animals, but they decline progressively and are almost absent in rats more than 6 months old, an age which would correspond to approxi-

‖ Blumenthal, H. T.: Unpublished data.

¶ References 13 and 15 to 19.

mately 20 years of age in man. On the other hand, the proportion of granular basophiles decreases slightly during the first month in the rat, and thereafter remains constant; the percentage of nongranular basophiles remains approximately constant throughout life. However, Payne [16] has observed that with advancing age in the chicken the mitochondria in the basophiles increase and become vesiculated, causing the cell to swell.

The spontaneous appearance of adenomas of the anterior hypophysis of senescent rats has been described by several investigators.# These adenomatous growths have been found almost entirely in the anterior lobes of old rats and some humans, and their presence is apparently a manifestation of advancing age. However, they probably have no, or only slight, secretory function, since the large majority are composed only of chromophobe cells; a few contain scattered eosinophiles, but even in these chromophobes are markedly predominant.

Colloid cysts at the junction of the anterior and intermediate lobes are common in older animals and, according to Strasseman and Krush,[24] appear in humans after about 40 years of age; they appear to arise from degenerative processes in anterior lobe cells and become progressively larger with increasing age. Further, Lansing and Wolfe [25] have described a progressive increase in fibrillar material with advancing age, and this is almost entirely reticular in character, found only in intimate relation with sinusoidal capillaries. An independent reticular meshwork for the support of anterior lobe cells was not found, nor was there a conversion of reticulum to collagen as commonly observed in the supporting structure of other organs with increasing age.

There are relatively few reports dealing with the levels of pituitary activity at various ages, and these are not in complete agreement. Saxton and Greene [26] have observed that pituitary glands from old female rabbits produce about the same degree of follicle stimulation and luteinization as young mature females; in contrast to the present observations, they have also reported less thyroid stimulation and generally greater stimulation of the adrenal cortex by the hypophyses of older animals. Saxton and Loeb,[7] utilizing human pituitaries implanted into immature female guinea pigs, also found no essential difference in follicle-stimulating and luteinizing effects between the glandular hypophyses of young and old persons; the latter group included women past the menopause and men between the ages of 60 and 90, as well as subjects of both sexes during the sexually active period of life. The thyroid glands of guinea pigs receiving implants of human pituitary from five persons between 60 and 90 years of age showed a greater degree of hypertrophy than in some of the guinea pigs receiving hypophyses from younger persons; similar results were obtained in the present experiments.

Marine, Rosen, and Spark [27] as well as Severinghaus,[28] have presented experimental data supporting the conclusion that thyrotropic hormone is elaborated by the acidophilic cells. Based on the progressive diminution in the number of acidophiles with age as noted above, a diminution in thyrotropic hormone content of the hypophysis would be anticipated in senescent persons. However, the observations of Saxton and Loeb,[7] as well as the present data, fail to support such a conclusion. Rather, they would indicate that thyrotropic hormone is secreted by the basophile cells, since the latter do not appreciably diminish with advancing age after puberty.

References 19 to 23.

Heinbecker [29] also maintains that the basophiles secrete thyrotropin; his conclusion is based on the observation that experimental injury of the hypothalamus in the dog results in degeneration of basophile cells and a hypoplasia of thyroid follicles.

The frequent association of basophile adenoma of the hypophysis and adrenal cortical hyperplasia in Cushing's syndrome strongly suggests that the basophile cells secrete corticotropin. However, Heinbecker [29] maintains that the latter hormone is secreted by the acidophiles and that the basophile adenomas of the hypophysis are nonsecretory. Nevertheless, the curve of corticotropic content of the human hypophysis with advancing age, including the higher content in the two youngest age groups in the present series, appears to be consistent with the changes in the relative number of basophiles during the life span. Consistent also with the concept that the basophile cells secrete corticotropin, is our observation that this hormone, like follicle-stimulating hormone, is not inactivated by formalin in concentrations of 1% or greater.

In general, the present observations, as well as those by Saxton and Loeb [7] dealing with the gonadotropic hormones, tend to support the statement of Hirsch [30] that valid evidence for hypofunction of the anterior pituitary in old age has not yet been presented. This appears to be particularly true of those tropic hormones which appear to be secreted by the basophile cells. Important also would be information on age changes in content of those hypophyseal hormones secreted by the acidophile cells, particularly growth hormone, in view of implication of that hormone in glucose metabolism and the high frequency of diabetes after approximately the age of 50.

It would appear from the foregoing discussion, therefore, that the diminution with age in target-organ function, at least in the case of the thyroid, adrenal, and sex glands, is either due to progressive failure in the delivery mechanism of the hypophysis or to inherent failure of the target organs. Structural alterations which occur in the connective tissue of the latter as age advances have been the subject of considerable investigation, and in some instances changes in the glandular elements have also been studied.* My studies have dealt specifically with the loss of potentiality for mitotic response in the thyroid, parathyroid and adrenal glands with advancing age, and the only partial recovery of this function with the administration of ovarian hormones.† In contrast to these observations in animals, Solomon and Shock [35] have reported that the ability of the adrenals of patients to respond to corticotropic hormone, as manifested by an eosinopenia, is not appreciably diminished in elderly persons. On the other hand, as regards thyroid function, while there is no appreciable increase in the incidence of myxedema after age 50, it seems well established that there is a gradual reduction in the basal metabolic rate in otherwise healthy persons after the age of 20 to 25.

The present observations relative to parathyrotropic activity of the human hypophysis deserve further comment and extended investigation. Despite gradually accumulating data on the parathyroid glands, much information is needed concerning factors involved in the control of their function. The influence of blood calcium and phosphorus levels on the activity of these glands is fairly well understood, but the hormonal control of parathyroid function is not well comprehended. Several lines of evidence indicate the possible existence of a parathyrotropic hormone of the hypophysis.‡ However, these observations are not supplemented with data on serum

* References 31 to 34.

† References 10 and 11.

‡ References 36 to 42.

calcium and phosphorus levels or adequate histological examination; on the contrary, cases of Simmond's pituitary cachexia do not, as a rule, show abnormal calcium or phosphorus levels.[43] Furthermore, Aschner [44] was unable to detect any change in the parathyroid glands following ablation of the hypophysis. Several investigators § have also described histological changes indicating increased activity of the parathyroid glands following the injection of hypophyseal extracts, but it is unlikely that preparations of established purity were employed, and the possibility cannot be excluded that the enlargement of the parathyroid glands may have been due to growth hormone. The same criticism is applicable to human cases of associated pituitary and parathyroid tumors, since many of these persons also exhibited signs of acromegaly. Such objections may particularly be applied to the experiments of Ham and Heist [45] who utilized a diabetogenic anterior pituitary extract; it is now well known that growth hormone has such a diabetogenic effect. Furthermore, the possibility cannot be excluded that the increased activity of the parathyroid glands in some of these experiments may have been mediated through other glands of internal secretions, particularly the thyroid, since there is evidence to support such a mechanism.‖

From the immediate foregoing, it is apparent that the existence of a parathyrotropic hormone of the hypophysis should be considered doubtful. On the contrary, several investigators have observed an inhibition of the parathyroid glands by the anterior hypophysis.¶ The present experiments indicate a possible parathyroid stimulating effect only in prepubertal persons; adult hypophyses produce an inhibition of parathyroid activity.

Finally, the experiments utilizing physical agents and chemical substances demonstrate a remarkable resistance of thyrotropic and corticotropic hormones to such influence. Further, there was no evidence that these agents in any way enhanced the activity of these hormones. Since the latter are either protein or polypeptide in nature, it is of particular interest that they retain activity even after treatment with denaturing agents such as heat (56 C.) or concentrated solutions of sodium sulfate. Various fat solvents also failed to reduce the activity of thyrotropin and corticotropin. Particularly noteworthy were the effects of formalin in concentrations of 1% or greater which appear to inactivate thyrotropin but not corticotropin. The peculiar resistance of the latter has also been demonstrated by Li [67] and by Brink and his co-workers,[68] who have reported that peptic digests of purified protein corticotropin are as active as the original material. Similarly, boiling the protein for short periods with dilute HCl also resulted in the appearance of smaller fragments which were active. Our observations on the effects of formalin are in agreement with those of Hayward, Pollock, and Loeb,# who observed that thyrotropin of cattle anterior pituitary is inactivated by formalin while the latter had no effect on follicle stimulating hormone. These observations suggest a chemical similarity between the latter substance and corticotropin. In general, the agents listed in Table 3 offer a variety of ways in which hypophyses may be stored in order to avoid effects of autolysis which probably account for the wide range of results obtained with untreated glands.

§ References 45 to 48.
‖ References 49 to 63.
¶ References 64 to 66.
# References 5 and 6.

SUMMARY

One hundred fourteen human hypophyses were implanted into an equal number of immature female guinea pigs. Mitotic counts performed on the thyroid, adrenal cortex, and parathyroid glands were utilized as a means of estimating thyrotropic, corticotropic, and possible parathyrotropic content. Neither the sex of the donor nor the disease causing death appeared to influence appreciably the hypophyseal content of these hormones.

Except for possibly the first two decades of life, there does not appear to be an appreciable diminution in content of corticotropin or thyrotropin with advancing age. With the possible exception of the youngest age group, no evidence was observed for the existence of a parathyrotropic hormone; on the contrary, these human hypophyses appeared to inhibit mitotic activity in the parathyroid glands of the test guinea pigs.

Thyrotropin and corticotropin appear to be resistant to a variety of chemical and physical agents, thereby affording a means of preserving hormone activities otherwise inactivated by autolytic processes in untreated glands.

Present observations are correlated with cytological and physiological studies by other investigators, and it is concluded that there is at least no evidence for a decrease in tropic hormone content in the hypophysis with advancing age of those endocrine substances which are produced by the basophile cells.

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HEPARIN IN EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS IN THE RABBIT

II. The Effect of Heparin on the Regression of Atherosclerosis

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AFTER the cessation of cholesterol feeding in rabbits, there is a gradual regression or healing of the cholesterol-induced lesions. Anitschkow [1] studied the problem in detail and found that the transformation of a large plaque, rich in lipids, into one composed of fibrous tissue took two to three years, and that even then small amounts of lipid remained. Studies in this department [2] have confirmed the fact that regression of lesions in cholesterol-fed rabbits is indeed a very slow process. During a period of observation of six months following cessation of cholesterol feeding there was no demonstrable tendency of the lesions to regress from the standpoint of either gross morphology or chemistry. The total amount of cholesterol per aorta remained unchanged, although there was a relative increase in the ratio of free to total cholesterol. A certain amount of reorganization and fibrosis of the lesions could be detected microscopically.

Spontaneous regression of cholesterol-induced lesions has been described in the chicken [3] and dog.[4] Some observers have suggested that the lesions of human atherosclerosis may undergo regression. Leary [5] described a "defense mechanism" which is purported to remove excess fat and cholesterol from the arteries in youth and from the ascending aorta even in old age. Observers in two world wars * have commented on the paucity of atherosclerotic lesions in persons who subsisted for a long time on starvation diets. Wilens [8] noted that undernourished persons had far less atherosclerosis at autopsy than did the obese. This was marked in those who had suffered from protracted undernutrition.[9] In the latter, resorption of lesions may have been a factor.

Attempts to influence the rate of regression of cholesterol-induced lesions in rabbits have been almost universally unsuccessful. Choline was believed to accelerate regression, as reported by Steiner [10] and Morrison and Rossi,[11] but more recent work † has failed to confirm this, and, indeed, suggested that choline may sometimes aggravate the lesions. Recently Stamler and co-workers [14] have reported that estrogens caused almost complete regression of atherosclerotic lesions in the coronary arteries of chicks, with no appreciable effect on the atherosclerotic lesions of the aorta. Both the lipid and the fibrotic components of the coronary plaques disappeared under the influence of estrogen. This work has not yet been confirmed.

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* References 6 and 7.
† References 12 and 13.

Heparin has been shown to affect the nature of the circulating plasma lipoproteins in man and in the cholesterol-fed rabbit. Graham and co-workers [15] found that heparin kept the development of giant lipoprotein molecules of the S_f 10 to 50 class down to 15 to 50% of the values in cholesterol-fed control animals. A single injection of sodium heparin intravenously in the cholesterol-fed rabbit produced dramatic changes in the lipoprotein spectrum characterized by a decrease in concentration of molecules of the higher S_f classes, and an increase in those of lower S_f classes. In a series of 20 pairs of rabbits fed 1 gm. of cholesterol three times a week for three to eight weeks, 10 to 25 mg. of heparin was given intravenously daily, and the injected animals were said by the authors to have been protected against the development of lesions.

In rabbits fed 1 gm. of cholesterol daily for 12 weeks and injected intramuscularly with 50 mg. of heparin daily we [21] have noted a definite reduction in the severity of atherosclerotic lesions as compared with control animals.

Because of its striking action on the plasma lipoproteins and its ability to retard the development of atherosclerosis in cholesterol-fed rabbits, it seemed worth while to determine whether heparin might also possess the ability to accelerate the removal of lipids from established atherosclerotic lesions in the rabbit.

MATERIALS AND METHODS

The animals employed were New Zealand white rabbits approximately 3 to 4 months of age at the beginning of the experiment and weighing 2.0 to 2.5 kg. For the first six weeks of the experiment 0.5 mg. of cholesterol in oil was added to the daily ration of ordinary rabbit food pellets of each rabbit, and this was increased to 1.0 gm. of cholesterol daily for the following six weeks. At the end of this 12-week period cholesterol feeding was discontinued and all animals received ordinary rabbit food pellets for the remainder of the experiment. After an additional seven weeks (19th week of experiment), the rabbits were divided into two groups matched as to sex and weight. There were 21 rabbits in the control group (13 males, 8 females), and 22 in the experimental group (13 males, 9 females). The rabbits in the experimental group were injected intramuscularly daily with 0.25 cc. (50 mg.) of heparin in gelatin-dextrose menstruum,‡ while the controls were not injected. Heparin treatment was carried on for 18 weeks and the animals were killed at the end of this period (37th week of experiment).

The dose of heparin used produced a marked prolongation of the coagulation time. In five rabbits injected with a dose of 50 mg. of heparin intramuscularly, coagulation time (measured by the capillary tube method) was prolonged three to five times at nine hours after injection and returned to normal values at 20 hours after injection. Tests for "clearing factor" were made using the method of Schwartz and co-workers [20] and none was demonstrated.

The animals were weighed at regular intervals, and blood was drawn for lipid analysis at 0, 12, 19, 25, 31, and 37 weeks. At autopsy, drawings of the extent of the aortic lesions were made on standard outline diagrams of the aorta. Lesions were graded visually as 0 to 4+ according to the following scheme: Lesions involving less than 2% of the surface of the aorta were graded 0. Lesions involving 2 to 10% were graded 1, while those involving 10 to 20%, 20 to 40%, and 40 to 80% of the surface respectively were graded 2, 3, and 4. The severity, as well as the extent, of the lesions entered into the grading.

In addition, the intima and media of the aorta were separated from the adventitia by dissection and extracted with absolute alcohol in a continuous extraction device for a period of eight hours. The tissue was then extracted with cold ethyl ether for another eight hours, and the extracts were pooled. The extracts were analyzed for lipid content using the standard methods employed in this laboratory for determination of serum lipids.§

‡ Supplied by The Upjohn Company, Kalamazoo, Mich.

§ References 17 and 18.

RESULTS

Body Weight.—The mean weight of the rabbits at the start of the experiment was 2,264.5 gm. They gained weight steadily for the first 19 weeks of the experiment, and at the time of separation into control and experimental groups the average weights in each group were 3,558.8 and 3,578.8 gm. respectively. Thereafter, the control animals continued to gain weight to a final mean weight of 3,668.4 gm., while the heparin-treated animals lost weight gradually to a final mean weight of 3,442.2 gm.

Blood Chemistry.—Table 1 shows the changes in the blood lipids, and the results of statistical analysis of differences between the control and heparin-treated rabbits. There was a marked response to cholesterol feeding with a rise in total cholesterol

TABLE 1.—*Summary of Serum Lipid Values: Mean Values*

	0 Weeks	13 Weeks	19 Weeks *	25 Weeks	32 Weeks	38 Weeks
Free Cholesterol						
Control	13.7	654.6	265.7	21.1	14.2	5.4
Heparin-treated	....	.....	.....	22.1	12.1	3.2
P	....	.....	.....	0.9	0.5	0.4
Ester Cholesterol						
Control	36.4	2262.3	824.5	74.4	31.2	45.0
Heparin-treated	....	.....	.....	70.6	42.3	40.0
P	....	.....	.....	0.9	0.2	0.5
Total Cholesterol						
Control	50.1	2950.4	1090.2	95.4	45.9	50.4
Heparin-treated	....	.....	.....	92.7	54.4	43.2
P	....	.....	.....	0.9	0.5	0.5
Lipid Phosphorus						
Control	5.5	27.6	19.3	4.3	3.8	4.1
Heparin-treated	....	.....	.....	4.7	3.7	4.3
P	....	.....	.....	0.7	0.4	0.6
Total Fatty Acids						
Control	11.3	72.29	32.8	7.5	5.8	5.6
Heparin-treated	....	.....	.....	8.6	5.5	6.0
P	....	.....	.....	0.5	0.6	0.4

* All animals were grouped together up to 19 weeks, at which time division into heparin-treated and untreated controls was effected.

to a mean value of 2,950..4 mg. per 100 cc. at 12 weeks. Lipid phosphorus rose to 27.6 mg. per 100 cc. and total fatty acids to 72.29 mEq. per liter. After cessation of cholesterol feeding, the serum lipids fell slowly to lower levels. At 19 weeks (7 weeks after cessation of cholesterol feeding) the mean total cholesterol content was 1,090.2 mg. per 100 cc., lipid phosphorus 19.3 mg. per 100 cc., and fatty acids 32.8 mEq. per liter. Heparin treatment was initiated at this time. At 25 weeks (13 weeks after cessation of cholesterol feeding), the total serum cholesterol of the heparin-treated animals was 92.7 mg. per 100 cc. and that of the controls was 95.4 mg. per 100 cc. The values for lipid phosphorus were 4.7 and 4.3 mg. per 100 cc. respectively, and for fatty acids 8.6 and 7.5 mEq. per liter. By the 32d week (20 weeks after cessation of cholesterol feeding) the lipid levels were back to normal. The administration of heparin did not appear to influence the rate of decline of the serum lipids or the final levels achieved. Inspection of the probability values shows that there were no significant differences in the serum lipid values between the heparin-treated animals and the controls.

Gross Morphological Gradings.—The distribution of the gross grades of aortic atherosclerosis is shown in Table 2. It is of interest that three of the heparin-treated animals showed little gross evidence of atherosclerosis, whereas all the controls showed moderate to severe grades of lesions. The aortic lesions in only four of the heparin-treated animals were graded as 4+, while this grade was assigned in 10 of the control animals. The mean of the grades for the heparin-treated group was 2.55, and the mean of the grades for the control group was 3.19. The difference between the means is just statistically significant at the 5% level.

TABLE 2.—*Morphological Gross Grading and Cholesterol Content of Aortas*

Control Group			Heparin-Treated Group		
	Aorta Cholesterol			Aorta Cholesterol	
Gross Grade	Free	Total	Gross Grade	Free	Total
4	29.6	52.9	4	18.3	26.4
4	16.9	28.3	4	23.8	42.4
4	—	51.5	4	30.2	43.2
4	13.9	20.8	4	7.1	10.1
4	14.5	21.0	3	23.6	38.7
4	44.1	57.9	3	—	—
4	21.9	25.3	3	17.5	25.1
4	6.9	13.3	3	18.0	20.5
4	17.0	25.3	3	3.9	6.8
4	29.6	42.0	3	9.7	14.8
3	11.0	18.3	3	15.1	25.3
3	12.2	22.1	3	13.2	14.3
3	7.0	11.6	3	15.1	22.2
3	10.2	15.4	2	4.7	9.1
3	14.7	24.5	2	4.4	10.0
2	1.2	2.9	2	8.9	14.2
2	5.2	8.7	2	5.4	7.9
2	6.1	9.9	2	12.0	18.9
2	7.1	11.6	2	6.2	10.2
2	3.5	10.3	1	0.2	0.2
2	3.4	7.1	0	0.6	0.5
			0	0.8	0.8
Mean 3.19	13.8	22.9	2.55	11.4	17.2
S. D. ±0.875	±10.7	±15.77	±1.14	±9.06	±12.84
S. E. ±0.196	± 2.46	± 3.5	±0.25	±2.03	± 2.95
P 0.05	> 0.9	< 0.3			

Lipid Content of Aortas.—In general there was a moderately good correlation between the chemical content of the aorta and morphological grading (Table 2).

The total cholesterol content of the aortas in the heparin-treated group ranged from 0.5 (No. T-72; Grade 0) to 43.2 mg. (T-95; Grade 4), with a mean value of 17.2 mg. The aortas in the control group ranged from 7.1 (T-88; Grade 2) to 57.9 mg. (T-87; Grade 4) with a mean value of 22.9 mg. Statistical analysis of the data indicates that the difference between the groups, with respect to free and total cholesterol content of the aortas, is not significant.

COMMENT

Heparin has been reported to retard the development of atherosclerosis in rabbits fed 1 gm. of cholesterol three times a week for three to eight weeks. The dose of heparin used was 10 to 25 mg. intravenously, and it was demonstrated that the action of heparin on the lipoproteins long outlasted its anticoagulant effect.[15] In

another experiment we have been able to demonstrate a retarding action of heparin on the development of atherosclerotic lesions in rabbits fed cholesterol for 8 to 12 weeks, without any appreciable difference in the quantitative levels of the serum lipids becoming apparent.[21] In the latter experiment, as in this one, heparin was given in high dosage by the intramuscular route, and there was marked prolongation of the clotting time. Our rabbits, like Anfinsen's,[16] failed to make any detectable amount of "clearing factor" following heparin injection. The ability to make "clearing factor" may perhaps be a property of some strains of rabbits only.

Inspection of the morphological severity of the atherosclerotic lesions suggested that heparin had some effect in promoting regression. Since grading is not linear, some objection might be raised to handling the data in this fashion. Chemical determination of cholesterol content per aorta would appear to be a more objective method of recording the severity of atherosclerosis. This measurement correlates fairly well with gross grading when large numbers of animals are used.[2] With individual animals, or small groups, the correlation is poor, as would indeed be expected since such factors as aortic area and varying relationship of morphological detail to chemical content of lesions must be considered. However, taken as a separate measure of the atherosclerotic process, it may serve as a useful index. Considered from the point of view of cholesterol content per aorta, there was no real difference between the heparin-treated and the control animals in this experiment.

Serum lipids failed to show any consistent quantitative changes that could be attributed to heparin administration. The rate of fall of serum lipids following the cessation of cholesterol feeding was not affected. Heparin is, however, known to produce profound changes in the physicochemical characteristics of serum.[15] But it is evident that the changes of this character that may have been brought about by heparin administration failed to influence the regression of the atherosclerotic lesions appreciably within a period of 18 weeks.

The cholesterol in the atherosclerotic plaques is not entirely excluded from the general turnover of body cholesterol. Experiments with tritium-labeled cholesterol have shown that there is a turnover of aortic cholesterol, but at a slower rate than of serum cholesterol.[19] It is important therefore to continue the search for substances that will influence the mobilization of cholesterol contained in atherosclerotic lesions.

SUMMARY AND CONCLUSIONS

Rabbits fed cholesterol in oil for 12 weeks (0.5 gm. daily for six weeks, and 1 gm. daily for six weeks) developed a marked lipemia and hypercholesterolemia. Following cessation of cholesterol feeding there was a gradual decline in lipid levels. Seven weeks after cessation of cholesterol feeding, half the animals received daily intramuscular injections of 50 mg. of heparin in a gelatin-dextrose menstruum for a further period of 13 weeks. The decline of lipid levels did not appear to be affected by heparin administration.

The severity of aortic atherosclerosis was graded visually, and by chemical estimation of cholesterol and lipid phosphorus content of the inner layers of the aortic wall. Visual grading suggested a lower degree of atherosclerosis in the heparin-treated animals, but this was not borne out by the chemical analyses.

Heparin in the dosage used in this experiment did not influence either the rate of regression of established atherosclerotic lesions or the rate at which the serum lipids regained their normal levels after the cessation of cholesterol feeding.

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LESIONS IN ACCESSORY SPLEENS

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A RECENT study of the occurrence of accessory spleens revealed an over-all incidence of 10%.[1] It appeared of interest, therefore, to investigate the lesions occurring in accessory spleens and to determine whether the changes occurring in them were identical with those in the main spleen.[2]

MATERIAL AND METHODS

The necropsy records of the Veterans Administration Hospital, Houston, Texas, were reviewed for the period Jan. 1, 1950, to Aug. 13, 1953, during which time 1,000 necropsies were performed (three on women, the rest on men). At all necropsies a search for accessory spleens was routinely made. Among the 1,000 patients examined, accessory spleens were located 94 times. This study concerns the accessory spleens of these 94 patients. The gross and microscopic features of the accessory spleen or spleens were compared with those of the main spleen.

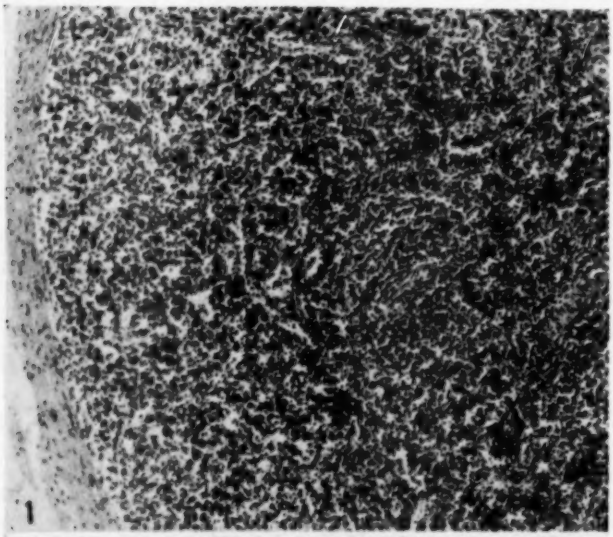
RESULTS

Of the 94 patients (70 white and 24 Negro) 76 had one accessory spleen, 15 had two accessory spleens, and 1 patient each had three, five, or "many" accessory spleens. The 76 solitary accessory spleens were located as follows: 66 at the hilus, 6 in the tail of the pancreas, 3 near the hilus, and 1 along the splenic artery 6 cm. from the hilus of the main spleen. Among the 15 patients with two accessory spleens, both spleens were located at the hilus in 12; in 2 patients one accessory spleen was in the tail of the pancreas and the other at the hilus; in 1 patient one was at the hilus and the other near the hilus. The three or more accessory spleens of the three patients all were located about the hilus or along the splenic vessels.

The smallest accessory spleen was 0.2 cm. and the largest 4 cm. in diameter. Most of them were between 0.5 and 1.5 cm.

In the 94 patients with accessory spleens, no lesions were noted in either the main or the accessory spleens in 26. In the remaining 68 patients the following changes were observed. There were varying degrees of chronic passive hyperemia in both spleens of 38 patients. In 28 the chronic passive hyperemia was due to cardiac decompensation, and in 10 it was due to portal cirrhosis of the liver. Increased hemosiderin deposit was observed in all spleens of nine patients who had received repeated blood transfusions (Fig. 1). A hyalin-like ground substance was noted in the Malpighian corpuscles and about the blood vessels of the spleens in three patients. Episplenitis was encountered in the spleens of two patients. Hemo-

From the Department of Pathology, Baylor University College of Medicine, and the Veterans Administration Hospital.



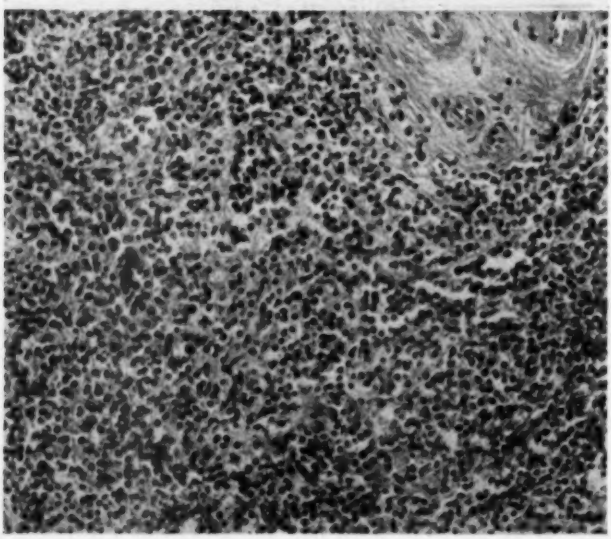


Fig. 1.—Accessory spleen of a Negro man, aged 44, who received multiple blood transfusions and died of pulmonary tuberculosis (N-138-53). Dark-brown deposits of hemosiderin are scattered throughout. The change is similar to that in the main spleen. (× 80)

Fig. 2.—One of two accessory spleens in a white woman, aged 65. Microscopic study of the organs disclosed the presence of multiple myeloma not suspected during life (N-175-51). The infiltration with plasma cells in the accessory spleen is similar to that in the main spleen. (× 200)

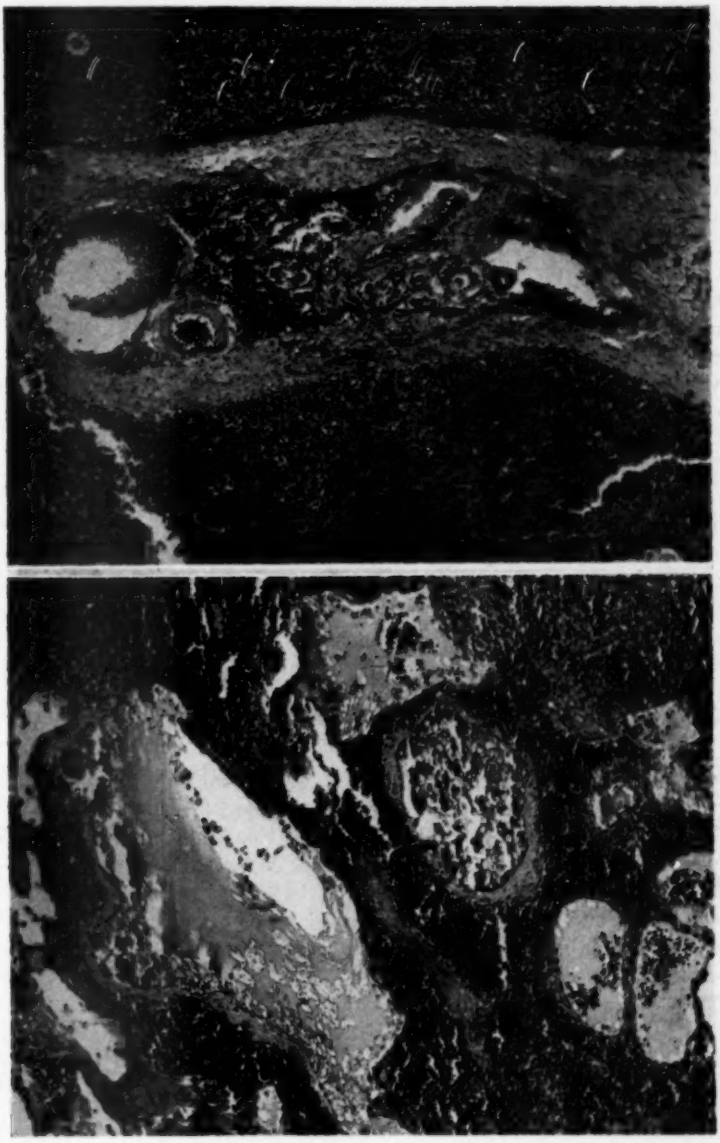


Fig. 3.—Accessory spleen of a white man, aged 30, with carcinoma of the lung (N-98-51). Groups of what are apparently islet cells are seen in a septum inside the capsule of the accessory spleen that was located in the tail of the pancreas. The lesion was not duplicated in the main spleen. (× 80)

Fig. 4.—The main spleen of the patient whose accessory spleen is shown in Figure 3. Spacious endothelial-lined spaces form an angioma beneath the capsule. This was not seen in the accessory spleen. (× 80)

poietic foci were noted in the spleens of one patient with anemia due to carcinoma of the lung and in one with macrocytic anemia, unclassified. In one patient each there were in both spleens acute splenitis, periarteritis nodosa, splenomegaly with Banti's syndrome, miliary tubercles, granulomatous nodules of undetermined etiology, and focal necrosis. In one patient each in both spleens there were identical lesions of reticulum cell leukemia, acute lymphocytic leukemia, unclassified leukemia, chronic granulocytic leukemia, and multiple myeloma (Fig. 2). In two patients with lymphoblastoma of the Hodgkin's type, characteristic lesions were observed in the main spleen and in the accessory spleens. In one patient with reserve-cell carcinoma of the lung, metastasis occurred in both spleens.

In two patients the lesions were not duplicated in the main and accessory spleens. In one patient with chronic passive hyperemia in both spleens, calcified spheroid bodies were in the main spleen but were not seen in the accessory spleen. In the other patient who had chronic passive hyperemia in both spleens, groups of what were apparently islet cells were noted inside the capsule of the accessory spleen in the tail of the pancreas (Fig. 3). In the same patient a small cavernous angioma in the main spleen was not duplicated in the accessory spleen (Fig. 4).

COMMENT

The main spleen and the accessory spleen or spleens, whatever their location may be,* have identical functions and are apparently subject to the same circulatory, hormonal, and other influences. It has been therefore widely assumed without substantial documentation that lesions occurring in the main spleen also occur in the accessory spleen. This is of practical moment when splenectomy is undertaken.[5] The evidence gathered herein supports the contention that lesions affecting the main spleen also affect the accessory spleen or spleens.

SUMMARY

A routine search for accessory spleens in 1,000 consecutive necropsies yielded 94. Thus this study reaffirms that the over-all incidence of accessory spleens is about 10%. In all other instances whenever there was a lesion in the main spleen the accessory spleens were similarly involved. Lesions not duplicated were noted in only two patients; in one, calcified spheroid bodies in the main spleen were absent in the accessory spleen, and in the other, groups of islet cells in the accessory spleen were not seen in the main spleen and a cavernous angioma in the main spleen was absent in the accessory spleen.

* References 3 and 4.

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RENAL CHANGES INCLUDING TOTAL CORTICAL NECROSIS IN CHOLERA

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THE BEHAVIOR of the kidney is a matter of great concern in cholera. Even the lay villager of India gives a sigh of relief when the patient passes urine after the day or two of anuria of the algid stage. The commencement of urinary flow heralds the termination of this stage and the beginning of the stage of reaction. This does not necessarily mean that the danger is over. Some of the cases die with full urinary output in the stage of reaction, while in a few others the quantity of urine gradually falls again although the stage of shock is over, the blood urea and nonprotein nitrogen increase, and death follows with typical signs and symptoms of uremia. This postcholeric uremia ends fatally usually between the 7th and the 10th day, sometimes earlier or later.

The pathological changes within the kidneys reported so far * have been too inadequate to account for the grave renal disturbances in cholera. In more recent years attention has been drawn to the possibility of intense circulatory disturbances within the kidneys in various surgical, obstetrical, and medical conditions, among which cholera has tentatively been included.† But at least so far as the cholera kidney is concerned, the available pathological reports cannot be regarded as giving clear positive evidence in favor of such a conception. While studying human kidneys from various pathological conditions, De and Sengupta [11] observed changes which were suggestive of the occurrence of cortical vasospasm and corticomedullary diversion of blood in cholera. The present investigation extends this work and adds further observations from recent cases of cholera.

MATERIALS AND METHODS

The kidneys from 14 cases (10 males, 4 females) of cholera dying in the stage of shock, of 10 cases (9 males, 1 female) dying in the stage of reaction, and of 8 cases (7 males, 1 female) dying of postcholeric uremia were available for study. None of the females were pregnant, nor had they recently passed through parturition. The diagnoses in all the cases of the first and third groups and four cases of the second group were confirmed bacteriologically. In the rest the intestinal lesions, the history, and the appearance of the stools were typical of cholera, although bacteriological confirmation was lacking. Full autopsy was possible in four cases of the first group, in all cases of the second group, and in five cases of the third group. Where full autopsy

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* References 1 to 6.

† References 7 to 10.

was not permitted, the kidneys were removed through lumbar incisions and the pedicle tied immediately after removal. One of each pair of kidneys was prepared for Pickworth's stain for demonstration of the vascular pattern as described in a previous paper.[11] Blocks of tissue including both cortex and medulla were taken from the other kidney and fixed in 10% formol-saline for paraffin sections, which were stained by Ehrlich's acid-hematoxylin and eosin method, by the periodic acid-Schiff technique for demonstration of glomerular basement membrane, and by a modified Highman method[12] for demonstration of iron by the Prussian blue ferric ferrocyanide reaction. Frozen sections were stained with Scharlach R for neutral fat. The Gram-Weigert method for fibrin was also used on some of the paraffin sections.

The urine of 25 cases of cholera confirmed bacteriologically was examined almost daily for evidences of renal damage, especially for albumin, casts, and red blood cells, and also for free hemoglobin in the supernatant fraction of centrifuged specimens. Ten control cases of noncholeric diarrhea and of acute bacillary dysentery with clinical evidence of dehydration were studied in a similar way.

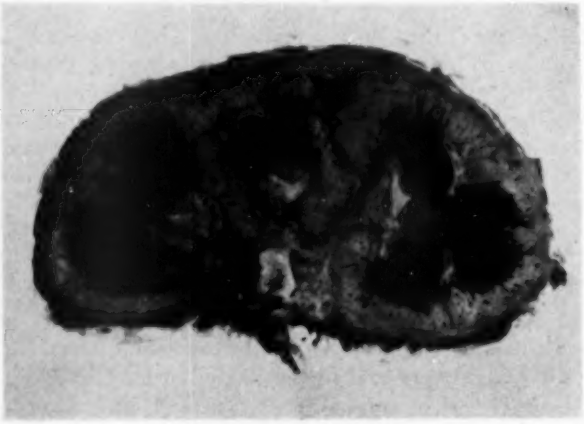

Fig. 1.—Cut surface of kidney showing cortical pallor and medullary congestion; interlobular vessels prominent. Stage of shock.

RESULTS

GROUP I: *Kidneys from Fourteen Cases Dying in the Stage of Shock.*—The stellate vessels beneath the capsule appeared injected, while the intervening areas looked pale. The cut surface of the kidneys showed evidence of pallor of the cortex with congestion of the medulla. The interlobular vessels stood out conspicuously against the pale background (Fig. 1).

Pickworth preparations disclosed cortical ischemia, which was irregular and patchy in distribution, being more obvious near the surface, in the middle, or near the boundary zone, either confined to the glomeruli or involving the intertubular capillaries. At some places the juxtamedullary glomeruli and their efferents were seen joining numerous engorged medullary vessels, while in other areas the latter could be seen emerging directly from the arcuate vessels. Edema was present in the connective tissue separating the medullary tubules. In two cases, one of which has been reported earlier,[11] the cortical ischemia affected most of the sections and sometimes the whole depth of the cortex, including the juxtamedullary glomeruli. In these cases medullary congestion was also more marked.

Parenchymatous changes included some degree of cloudy swelling of the epithelium of the cortical tubules and, in two kidneys, fatty and necrotic changes in the tubules.

GROUP II: *Kidneys from Ten Cases Dying in the Stage of Reaction.*—The cut surface and the outer surface exhibited a uniform hue, and no distinction could be made between the cortex and the medulla from their color. The tubular epithelium showed cloudy swelling in some cases. In one case fatty change in the epi-

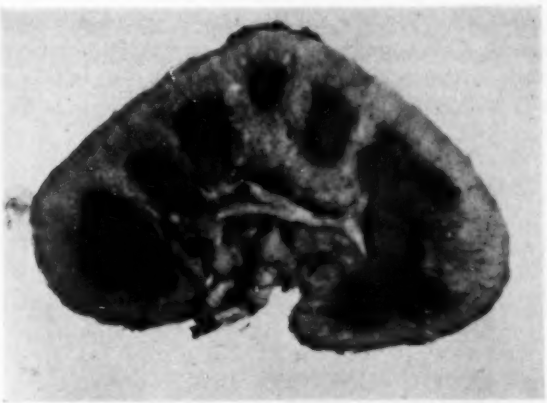

Fig. 2.—Kidney in postcholeric uremia; same features as in Figure 1; interlobular vessels obscure.

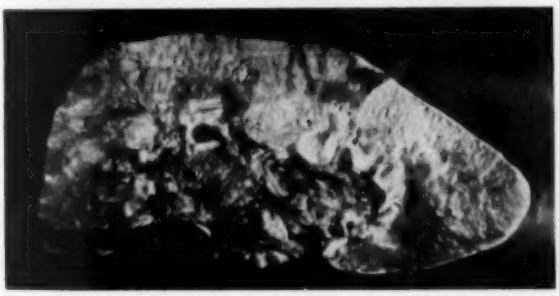

Fig. 3.—Cut surface of kidney showing multiple areas of cortical necrosis extending to surface. Postcholeric uremia.

thelium of both the cortical and the medullary tubules of one kidney was noted, and this was associated with a focal noncaseating tuberculous lesion in the boundary zone.

GROUP III: *Kidneys from Eight Cases of Postcholeric Uremia.*—Gross evidence of cortical pallor and medullary congestion was present in all of these kidneys. This color difference was usually more marked than in the kidneys of the first group, and the markings of the interlobular vessels were rather obscure (Fig. 2). Small (1 to 2 mm.) circumscribed pale nodules were scattered irregularly over the cortex (Fig. 2) in one case. Larger white and opaque areas of frank cortical necrosis (Fig. 3) were seen in both the kidneys of two cases and in one kidney

only of another case. The gross necrosis was patchy in distribution, and the patches were extremely variable in size. The smaller ones were pyramidal in shape with their bases on the surface and did not involve the whole cortical thickness. The larger areas did not spare the most superficial or the deepest zone of the cortex. The juxtapelvic regions of the columns of Bertini and the juxtamedullary regions of the intervening areas were involved in the larger necrotic patches. The areas of necrosis were clearly demarcated from the adjacent healthier areas by zones of congestion. The medulla as a whole was markedly congested, while the main cortical bed looked pale. Nowhere did the necrosis appear to extend into the pyramids or the capsule. The opaque, white necrotic patches, often showing evidence of breaking down in the more central parts and marked out by congested rims, were also clearly visible on the outer surface after the capsule had been stripped (Fig. 4).

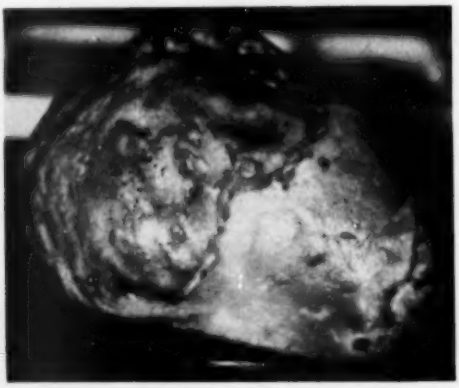

Fig. 4.—Appearance of outer surface of kidney shown in Figure 3. The capsule has been stripped off.

In hematoxylin and eosin preparations, many of the glomeruli appeared bloodless, although a few were congested. Cloudy swelling and necrosis of epithelium of the cortical tubules were constant features. There was no indication of selective damage to the upper or lower nephrons, nor was there any evidence of tubulovenous communication. Hyaline and granular casts were often found both in the cortical and in the medullary tubules. One case exhibiting hemoglobinuria during life showed yellowish-brown pigment casts within the convoluted tubules. In five of the eight kidneys the tubules contained breaking-down amorphous material, which gave a positive Prussian blue reaction. In two cases the renal stroma showed focal collections of mononuclear cells and fibrosis, suggesting repair of damaged tissues. Similar areas could also be made out around some glomeruli and tubules, especially in the boundary zone. One of these two kidneys exhibited small pale nodules scattered over the cut surface of the cortex on gross inspection. The Malpighian corpuscles frequently displayed periglomerular condensation of fibrous tissue, swelling and proliferation of the epithelium, and granular eosinophilic debris in the capsular space, which was either enlarged or almost obliterated.

There were also areas showing regenerative hyperplasia of tubular epithelium, sometimes forming syncytial masses. The retrogressive as well as the regenerative changes were confined to the cortical zone.

In the kidneys with total cortical necrosis, even the most superficial layers of the cortex were involved. The glomerular outlines were preserved, but the architecture of the tuft was lost. There was no evidence of dilatation or engorgement of blood vessels anywhere in the necrotic zone, and no outline of red cells, either distinct or obscure, could be made out on the arterial side. The outer walls of the roots of the interlobular arteries looked smudgy and were infiltrated with disintegrated leucocytes spreading out into the necrotic areas around. In one of these

Fig. 5.—Complete cortical ischemia; even justamedullary glomeruli bloodless; vasa rectal distended and full. Stage of shock. Pickworth's stain; 150 μ thick; × 25.

cases the lumen of these segments of the vessels contained a mass of eosinophilic material with a few scattered nuclear elements, but no fibrin could be demonstrated anywhere in the dead areas, even with special stains. In the other cases these blood vessels were empty. Around the areas of necrosis all the blood vessels were engorged and the stroma was infiltrated with lymphocytes. Fibroblastic proliferation was not pronounced. Both in the border area and in the healthier areas beyond, the tubules showed evidence of cloudy swelling and necrosis with no attempt at regeneration.

In all eight cases there was evidence of thickening and splitting of the basement membrane of the glomeruli in the periodic acid-Schiff preparations. French [18] regards this type of change as a sign of anoxic glomerular damage. The tufts seemed to be devoid of epithelial elements. Elongated cells, staining a purplish color and joining up at their ends with the thickened basement membranes, were

conspicuous in place of the epithelial cells. The tubular basement membrane showed no change except in areas of gross necrosis, where the reticulin framework of the cortex was completely destroyed.

The fat stain revealed the existence of fatty change in patches of cortical tubular epithelium in all the kidneys of this group. The tubules around the juxtamedullary glomeruli were not spared. The medullary tubules were, however, conspicuous by the absence of any trace of fat. Areas of gross necrosis also showed marked fatty change, which involved a few glomeruli as well. The epithelial cells of Bowman's capsule and the lining cells of some of the tufts were filled with globules of fat of small size, but blood vessels were not affected.

Fig. 6.—Patchy and incomplete cortical ischemia. Postcholeric uremia. Other details as in Figure 5.

Pickworth preparations revealed features of vascular pattern similar to those of the kidneys of the first group, but evidence of cortical ischemia was more frequent and more pronounced, especially in kidneys with total cortical necrosis. The zone around the necrotic patches in the latter was markedly congested. The glomeruli within the necrotic patches and the intertubular capillaries were almost devoid of blood. Those near the deeper parts of the larger necrotic areas filled well, possibly as a result of reflux from the venous side. Neither these capillaries nor the ischemic juxtamedullary glomeruli appeared to have any connection with the engorged vasa recta of the subjacent medulla.

The limited study of urinary changes revealed that after an attack of cholera excretion of albumin and casts is a constant feature and continues for 3 to 17 days. Hematuria lasted for 0 to 17 days—only 3 out of 25 cases did not pass any red blood cells, while in 2 cases they were detected only on the first day. The degree and duration of these urinary abnormalities did not depend upon the dura-

tion of anuria (14 to 48 hours) in the stage of shock. In three cases the first sample of urine voided after the period of suppression showed evidence of hemoglobinuria and the other urinary changes were also most pronounced. Two of these latter cases died later of postcholeric uremia, and the excretion of blood, albumin, and casts was continuous and progressive before death. In the third case the urine quickly became normal and the patients survived. In noncholeric diarrhea there was no evidence of anuria except in two cases, in which it lasted for 12 and 36 hours, respectively. Albuminuria was present and continued for one to five days. A few red cells and casts were seen for two to four days only in the two previously anuric cases.

COMMENT

The relative cortical pallor and medullary congestion in the cholera kidney did not escape the notice of Cohnheim,[1] who described a pale-gray to yellowish-gray opaque cortex and a bluish-red medulla. Our Pickworth preparations reveal its signficance and suggest that the process of vasoconstriction is limited to the cortical zone of the renal circuit. It is known ‡ that the walls of the medullary blood vessels are deficient in muscular elements. This is possibly the reason why they seem to offer a ready passage to the renal blood in face of cortical vasospasm. The juxtamedullary glomerular channel helps in this by-passing mechanism, but when the cortical ischemia is extreme, this is closed down and the blood enters the medulla from the arcuate arteries directly through the arteriae rectae verae. It thus appears that part of the cortical blood is diverted to the medulla, as in the animal experiments of Trueta and co-workers § and of De and co-workers || with cholera toxin. This corticomedullary diversion of blood, however, is nothing more than the expression of a stage in the progressive constriction of the renal vasculature [20] and does not fully explain the anuria, as the cortical ischemia was often incomplete. The more frequent absence of parenchymatous changes in the kidneys in the face of such grave disturbance of renal circulation for about 24 hours is surprising. Smith,[14] however, has suggested that if the ischemia is incomplete, a mere trickle of blood is sufficient to prevent anoxic damage of the kidneys for an indefinite time.

Most of the kidneys of the first group did not show any evidence of morphological damage. In two cases, however, necrotic changes in the tubular epithelium were seen and signs of cortical ischemia were marked. Besides, the presence of albumin and red blood cells in the urine was a frequent observation after this stage was over. Despite variations in degree, these findings indicate the occurrence of rather severe glomerular damage in the stage of shock. Similarly, it is found that while most of the cholera cases have uneventful recovery after the shock is over, a few of them later die of uremia following a return of progressive oliguria and anuria. In the present investigation, eight such cases were available for study. The kidneys exhibited a widely variable pathological picture, ranging from simple epithelial necrosis and fatty change, tubular regeneration, and glomerular reparative changes, to a gross picture of bilateral cortical necrosis. Necrosis and fatty degeneration of tubular epithelium and thickening and splitting of glomerular basement membrane were, however, constant findings. It is probable that the damage

‡ References 10, 14 to 16.
§ References 10 and 17.
|| References 18 and 19.

seen in these latter kidneys had been incurred during the first stage and that the two cases showing severe renal changes in this stage would have developed postcholeric uremia if they could have survived the initial shock. Cortical localization, both of the ischemia and of the renal lesions, suggests a causal relation between the two. Selective action of cholera toxin on the cortical tubules alone cannot account for the findings, as De and co-workers ¶ have shown experimentally that the medullary tubules are vulnerable to the toxin. Cohnheim [1] regarded the fatty change and the urinary abnormalities as the outcome of intense circulatory disturbance in cholera.

It appears from our observations that an attack of cholera regularly produces ischemic damage of the renal cortex. Usually the damage is mild and is manifested simply by albuminuria and hematuria, but in a few cases it is severe enough to produce obvious morphological changes, which favor the subsequent development of uremia. Factors that induce severe damage and severe ischemia in a minority of cases and not in others are obscure at the moment. But it is interesting to observe that three cases having copious hematuria in the postschock stage exhibited evidence of hemoglobinuria in the first sample of urine passed during recovery from shock, and two of them developed fatal postcholeric uremia. We think that hemolysis may play an important role in the development of renal lesions leading to postcholeric uremia. Cohnheim,[1] too, had envisaged the possibility of serious injury to red blood cells in cholera. We have also noted the occurrence of hyperbilirubinemia and an indirect positive van den Bergh reaction with or without hemoglobinemia or hemoglobinuria.# Although intravenous injection of hemoglobin in normal animals is not followed by renal failure,* it has been found to give rise to severe disturbance of renal function † in the presence of acidosis, dehydration, and shock, all of which prevail in cholera. Mason and Mann [21] and Reid [22] have demonstrated the occurrence of intense renal vasoconstriction in the presence of free hemoglobin in the plasma, and Corcoran and Page [23] have recorded the same with degradation products of hemoglobin. It appears probable that the renal vasoconstriction induced by the shock itself is rendered extreme in the presence of simultaneous hemoglobinemia. The renal parenchyma degenerates and dies as a result of the more complete cortical ischemia arising from a combination of these two factors. But the possibility of other, complementary factors cannot be excluded with certainty at the moment.

TOTAL CORTICAL NECROSIS

The occurrence of total cortical necrosis in three male cases of postcholeric uremia deserves separate comment. One of the cases died 21 days after the attack of cholera and showed gradual diminution of urine with a rise in blood nonprotein nitrogen before death. The other two cases died with anuria on the seventh day. The blood nonprotein nitrogen was not estimated, but postmortem evidence of gastritis and colitis suggested the uremic condition. A fourth case, showing opaque, white healing spots, probably represented a mild example of cortical necrosis. It may be observed here that Dunn and Montgomery [24] drew attention to the relative

¶ References 18 and 19.

References 21 and 22.

* References 23 to 27.

† References 27 to 30.

frequency of diarrheal conditions associated with this renal lesion, which has also been described in hog cholera,[35] though not in Asiatic cholera. The absence of true thrombi, the ischemia of the necrosed tissues, the involvement of the most superficial layers, and escape of the pyramids in our cases indicate the important role of cortical vasospasm in the genesis of the necrosis. In fact, heavy engorgement of the necrotic areas with blood was the chief factor which led Scriver and Oertel [36] to consider vasospasticanemia an improbable cause of the necrosis and to advance a more complicated explanation. Their doubt about the ability of vasospasm to bring about necrosis has been resolved by the experimental work of Byrom,[37] who observed maintained cortical pallor leading to cortical necrosis in rats after injection of vasopressin. It may further be noted that Byrom did not find any thrombus in the cortical vessels. However, we agree with Duff and Moore [38] that all instances of cortical necrosis may not have a similar pathogenesis and that the succession of events which lead to it may differ in different conditions.

SUMMARY AND CONCLUSIONS

The cholera kidney in the stage of shock shows some evidence of incomplete and patchy cortical ischemia and of medullary congestion. Usually the parenchyma escapes damage, but occasionally there may be necrosis and fatty change in the epithelium of the cortical tubules. No evidence of cortical change or vascular disturbance is found in the kidney in the stage of reaction. The kidneys of postcholeric uremia show signs of cortical ischemia and of medullary congestion which are more marked than in the stage of shock. Necrosis and fatty change of the cortical tubules and thickening and splitting of the glomerular basement membrane are constant features. Besides, evidence of repair and of total necrosis of cortical substance may be found in some specimens. The medulla is uniformly congested and is free from any sign of damage.

Reduction of renal blood flow aided by some degree of cortical vasospasm is thought to bring about a complete cessation of urinary secretion in the stage of shock. These factors are, however, usually incapable of causing any morphological renal damage, although functional damage is indicated by the occurrence of albuminuria and hematuria in the following stage. In a minority of cases, the occurrence of hemoglobinemia is a possible factor in intensifying the renal vasospasm and in rendering more complete the cortical ischemia, which is responsible for serious structural damage leading to postcholeric uremia. The lesions of total cortical necrosis seen in some of these latter cases are thought to be the extreme result of cortical vasospasm and ischemia.

Prof. G. R. Cameron, F. R. S., University College Hospital Medical School, London, gave help and criticism; Dr. A. K. Duttagupta, Superintendent, permitted publication of the cases, and Mr. M. Mazumder took the photographs.

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FAILURE TO PRODUCE RHEUMATIC FEVER IN RABBITS BY PROLONGED AND INTENSIVE STREPTOCOCCUS INFECTION

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IT WAS CONSIDERED important to investigate tissue changes in rabbits extensively subjected to Streptococcus infections in order to determine whether lesions closely simulating human rheumatic, renal, or cardiovascular diseases might be encountered. While much previous work on Streptococcus infections in animals has proved essentially barren, the possibility of rheumatic lesions occurring under quantitatively intense Streptococcus stimulation appeared to warrant further study.

Clinically and pathologically, rheumatic fever and rheumatoid arthritis present the appearance of fluctuating, chronic infectious diseases. An effort was made to induce similar prolonged and continuing infections among rabbits by injections of alpha and beta hemolytic streptococci, of staphylococci, and of various combinations of them in doses designed to yield sublethal but severe infections. Human disease patterns, such as true Aschoff bodies, were not encountered among the 280 rabbits studied, although interesting variations in the production of chronic diseases were noticed and a few rabbits exhibited a severe granulomatous myocarditis.

MATERIALS AND METHODS

Rabbits.—Albinos of mixed breeds and both sexes were purchased from one commercial source when the rabbits were approximately 2 to 6 months of age. They appeared healthy on arrival, were housed two in a cage, and were given a normal stock diet of rabbit pellets and water. During the latter part of the experiments, rabbits were individually caged.

Micro-organisms.—The following types of Group A beta hemolytic Streptococcus pyogenes were employed: 10 (NY-5 Strain), 12 (Strain "Nass," supplied by Dr. C. H. Rammelkamp Jr., who isolated it from a patient with acute nephritis), 14 (Strain "Fontaine," isolated from a patient developing rheumatic fever), and 19 (isolated from a patient with scarlet fever). Two alpha hemolytic Streptococcus strains were employed: one (α-1) was isolated from the throat of a person having a cold, and the other (β-2) was isolated from the throat of a person with acute rheumatic fever. A Group C Streptococcus (C) that was pathogenic for guinea pigs and that readily produced large, stable capsules, was also employed. Two Staphylococcus strains served as controls. One (S-1) produced pigment typical of Micrococcus pyogenes var. aureus. It was isolated from a normal person's nose, was markedly hemolytic on sheep, rabbit, or human blood agar, was coagulase-positive, and was inhibited either by 0.6 γ of chlortetracycline or by 0.08 unit of penicillin per milliliter of medium. Another strain (S-2) was isolated from a normal person's throat, was devoid of pigment, and was not hemolytic for sheep, rabbit, or human red blood cells.

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All organisms were grown in brain-heart infusion broth containing added 0.3% neopeptone (Difco) at 37 C. incubation for 15 to 18 hours, with storage at 4 C. a few hours unless the culture was immediately used. Growth was generally luxuriant, and approximately 10^8 viable forms per milliliter were counted when occasional checks were made, with use of broth dilutions and pour plates. Purity of cultures was checked by smears and by culture on blood agar.

After initial immunization with small (0.01 to 0.05 ml.) intracutaneous injections of viable organisms, doses were progressively increased and given subcutaneously, intramuscularly, and intravenously. Maximum single amounts per kilogram of body weight were 1 ml. of the entire, well-mixed culture. Owing to wide variations in pathogenicity and ability to cause continuing disease, injections varied among different treatment groups. As a rule, injections were given twice a week; if severe illness began, they were withheld or decreased in amount, and at the end of several months' observation treatments were discontinued a month to see if recovery ensued. Micro-organisms were administered to produce as severe an illness as possible that would be

TABLE 1.—*Summary of Treatment Groups in Relation to Number of Rabbits, Duration of Treatment, and Extent of Carditis*

Treatment	Number	Mean Days Duration	Mean Score Carditis
None	20	151	4
Streptococcus			
Group A			
Type 10	15	176	11
Type 12	12	138	11
Type 14	27	160	11
Type 19	10	144	15
Types 10 and 14	11	182	9
Group C	25	85	10
Alpha 1	9	204	10
Alpha 2	10	185	8
Alpha 1 and Type 14	14	158	17
Alpha 1 and Type 12	8	144	14
Alpha 1 and Type 19	37	114	15
Alpha 2 and Type 12	5	114	15
Alpha 2 and Type 19	37	150	16
Staphylococcus 1 (aureus)	20	105	7
Staphylococcus 2 (albus)	10	97	3
Staphylococcus 2 and alpha 1	10	117	2

nonfatal, and that would continue at that level for a maximum time. Table 1 outlines the various treatment groups in respect to the numbers of animals in each and average days' duration of experimental observation.

Observations.—Ainmals were examined daily and weighed twice weekly. Observations on 175 rabbits were made up to six months and the remainder were studied from 6 to 12 months. Occasionally blood was drawn from the femoral vein or artery for antistreptolysin O titration or for culture, and from a marginal ear vein for leucocyte counts. Cardiac puncture was done only at the time of killing, which was by air embolism, and blood taken then either from femoral vessels or from the heart was cultured in one or more of the following media: thioglycollate glucose broth, brain-heart infusion broth, or blood agar.

A complete postmortem examination was performed, which included weighing the heart, spleen, adrenals, kidneys, and liver. The following organs, in addition to sites of gross abnormality, were routinely examined microscopically: brain, kidneys, heart, lungs, adrenals, spleen, gonads, appendix, ileum, liver, and femoral marrow. Tissues were generally fixed in formol, embedded in paraffin, and stained with hematoxylin and eosin. For certain special studies,[1] the following stains were employed: Gram's, Giemsa, methylene blue, basic carbolfuchsin, and the Levaditi and various other silver stains.

Tissue sections were grouped into those from one rabbit and reviewed several times, including one study without reference to treatment received or gross pathology noted. A standard form was prepared on which various anatomic alterations were scored from 0, when normal, to

TABLE 2.—*Summary of Results in Rabbits Infected in Various Ways*

Treatment	Weight Loss	Chronic Disease	Arthritis	Carditis	Nephritis
Streptococcus					
Group A					
Type 10	+	++	++	++	+
Type 12	+	++	++	++	+
Type 14	+	++	++	++	+
Type 19	+	++	++	++	+
Types 10 and 14	+	++	++	++	+
Group A and alpha	+	++	++	+++	+
Group C	—	—	—	+	—
Alpha 1	—	—	—	—	—
Alpha 2	—	—	—	—	—
Staphylococcus 1 (aureus)	++++	++++	++++	+	++++
Staphylococcus 2 (albus)	+	+	—	—	+

TABLE 3.—*Summary of 280 Rabbits in Regard to Sex, Duration of Study and Incidence of Naturally Occurring Disease Often Termed "Encephalitozoon Cuniculi" Infection in Respect to Carditis*

	Number	Per Cent	Mean Score Carditis
Males	224	80	11
Females	56	20	14
Total	280	100	12
Males			
EC * +	67	30	16
EC —	157	70	8
Females			
EC +	27	48	20
EC —	29	52	8
Total			
EC +	94	34	17
EC —	186	66	8
Observed Under 180 Days			
EC +	37	21	25
EC —	138	79	9
Total	175	62	
Observed Over 180 Days			
EC +	57	54	13
EC —	48	46	7
Total	105	38	

* EC, Encephalitozoon cuniculi.

numerical recording of 1 to 4, depending on the extent of abnormality present. A final appraisal was made of the entire gross and microscopic pathology, in which such things as autolysis, complicating secondary infection (for example, intestinal coccidiosis with Pasteurella terminal septicemia), or other conditions were evaluated. This final evaluation gave a composite score for carditis which permited comparisons, as shown in Tables 1 and 3.

RESULTS

Intensive and prolonged infections with the streptococci and staphylococci employed did not produce rheumatic carditis, and true Aschoff bodies were not encountered. In general, the results of Group A Streptococcus infection were similar to those previously reported,[2] in which the A-10 (NY-5 strain), A-11 (Blackmore strain of Todd), and the A-no type (#98 strain of Rammelkamp) were given alone or in various combinations and were grown in a similar manner. The carditis was less severe than that observed when special young cultures of the NY-5 Streptococcus were administered [2] and did not manifest as much collagenic alteration or histiocytic hyperplasia.

Interesting variations in ability to produce chronic, continuing disease were noted, as generally summarized in Table 2. All the Group A streptococci were essentially similar in being able to produce a rather extensive, subacute infection characterized by arthritis, weight loss, adrenal hyperplasia, and focal septic carditis. This infection manifested moderate inertia, or what might be termed a flywheel effect, in that illness would continue a week or so after the last injection of streptococci. By contrast, the Group C Streptococcus had almost no residual or continuing effect, and even after several months of repeated sublethal injections the rabbits would either recover within a few days or else succumb to a rapid septicemia. It was impossible to produce chronic infection with the Group C Streptococcus. The severest chronic infection was caused by the Staphylococcus aureus, S-1. This organism gave rise to progressive emaciation, very severe septic arthritis, and extensive pyelonephritis. Rabbits would continue to decline in health for two or more months after the last injection of S-1 organisms. By contrast, the Staphylococcus albus, S-2, caused very little chronic disease and nephritis, while the alpha streptococci, a-1 and a-2, were essentially innocuous (Table 2).

Combinations of two different types of Group A Streptococci, 10 and 14, were not significantly different from the effects of either one alone. Among rabbits receiving both Group A beta hemolytic and alpha hemolytic streptococci, there were occasional instances of increased myocardial injury, with myocardial degeneration and focal necrosis, and an associated chronic passive congestion, with enlargement of liver and spleen. However, these changes were not impressive in all rabbits within the treatment groups, and it was impossible to conclude that there were differences between rabbits given the combined streptococci and those given only the Group A organisms which were significant. There were no differences in chronicity of disease among rabbits receiving Group A streptococci and those getting additional alpha streptococci.

The Group A Type 12 Streptococcus, presumably implicated in human glomerulonephritis,[3] did not produce nephritis in rabbits different from other Group A strains. This nephritis was primarily minor in extent and consisted of increased glomerular cellularity, tubular degeneration, and slight interstitial inflammation. It was difficult to evaluate in the presence of a naturally occurring infection which involved one-third of the animals, and which caused focal interstitial inflammation in the outer part of the renal cortex. This disease will be described in detail elsewhere.[1] It led to increased renal inflammation when organisms were experimentally administered. The Staph. aureus gave rise to the severest nephritis observed; all rabbits receiving this S-1 organism had extensive, chronic, focal pyemic lesions in

the kidneys. The Staph. albus, S-2, caused minor renal changes of a similar qualitative type, but only after more prolonged and extensive intravenous injections of organisms.

Subacute bacterial endocarditis with friable vegetations containing bacteria was seen in three rabbits, two receiving A-12 streptococci and one receiving the S-1 Staph. aureus. Small foci of bacteria associated with necrosis were commonly encountered in the hearts of rabbits dying with a septicemia, as in those given the Group C Streptococcus or others which occasionally received too large a dose of bacteria.

Two rabbits out of the 280 studied manifested an extensive, chronic carditis. Both appeared to have the naturally occurring systemic infection previously mentioned, which has often been termed Encephalitozoon cuniculi infection.[1] One rabbit had been given 12 injections of the A-12 Streptococcus during 219 days of observation. It had an initial weight of 2,225 gm., a peak of 2,545 gm. at Day 148, and thereafter a decline to a terminal weight of 2,375 gm. This rabbit had an extensive granulomatous myocarditis with acute and chronic inflammation, areas of fibrinoid degeneration, focal arteritis, round cell infiltration, and some chronic passive congestion. The other rabbit received 26 injections of the α-1 Streptococcus, which in other rabbits was essentially innocuous, during the 210 days of the experiment. It steadily gained weight, from 2,120 to 4,555 gm. There were foci of chronic to acute myocarditis with an appearance similar to that of the other animal described. Aschoff bodies were absent, but the fibrinoid degeneration, myocardial injury, and chronic inflammation resembled human rheumatic fever. Both animals were without treatment a month prior to death; yet there was continued active myocarditis, indicated by acute inflammatory foci of lymphocytes and other round cells amid acute degeneration of cardiac muscle fibers and collagen. The increased incidence of carditis in rabbits manifesting this natural infection [1] is summarized in Table 3.

The antistreptolysin O titers of rabbits given the Group A streptococci were elevated similarly to those previously reported.[2] Rabbits receiving the Group C Streptococcus, alpha streptococci, or staphylococci did not have elevated antistreptolysin O titers. White blood cell counts were made 186 times on 61 different rabbits during the last six weeks of observation. No significant differences were observed among treatment groups consisting of Group A and/or alpha Streptococcus and the Staph. albus, S-2.

In spite of extensive disease associated with the Streptococcus or Staphylococcus infections, there were no indications of degenerative arterial diseases comparable to human arteriosclerosis. Renal arteriolar necrosis or sclerosis of the type seen in malignant hypertension was absent. The arthritis in all cases was of a septic nature and was not similar to rheumatoid arthritis.

COMMENT

The extensive infections in rabbits produced during this study did not yield lesions resembling human diseases. While the repeated inoculations of streptococci were highly artificial counterparts to the clinical Streptococcus diseases associated with the onset of rheumatic fever, they permitted some evaluation of bacterial factors which by themselves appeared incapable of producing rheumatic fever, rheumatoid arthritis, glomerulonephritis, or human types of vascular disease. The obvious differences in immunity under the circumstances of these experiments could account

for failure to simulate more closely human diseases in this study, although immune factors alone do not necessarily explain the situation. Another potentially important factor might be the synergistic effects of streptococci and unknown infectious agents. This is suggested by the essentially fortuitous observation of certain lesions suggestively resembling rheumatic carditis in a few rabbits among those having both the naturally occurring systemic disease [1] and additional Streptococcus infection. The extensive studies in rheumatic fever by Pribram [4] and Menzer [5] likewise suggested the possibility of such synergisms.

In judging the rheumatic nature of experimental carditis, it was necessary to adhere strictly to the criteria for a true Aschoff body that Saphir and Langendorf [6] reported. While approximations to this goal are judged with risk and uncertainty, they are useful guides. In that regard, multiple types of Group A Streptococcus infections did not yield more "rheumatic" lesions than single types, contrary to the reports of Swift and Murphy.* Likewise, results of this study were similar to previous observations [2] of less severe experimental Streptococcus infections, and the lesions were not so "rheumatic-like" as lesions seen in rabbits given a young culture of NY-5 streptococci grown in a special blood medium.[2]

In future studies concerning the pathogenesis of rheumatic fever, it would appear more fruitful to investigate situations other than extensive Streptococcus infections in animals. A study of possible synergisms among infectious agents might be more rewarding than studies of the immune response in rheumatic persons, since little has been done in the former field and it could be approached with relatively less difficult techniques.

SUMMARY

Intensive and prolonged infections of rabbits by various streptococci and staphylococci did not yield lesions closely resembling those present in human diseases. It was not possible to produce satisfactory counterparts to rheumatic fever, rheumatoid arthritis, glomerulonephritis, arteriosclerosis, or arteriolonecrosis.

Multiple strain Streptococcus infections did not differ from single type infections. All the Group A streptococci studied were similar in producing a rather chronic, severe disease; the Group C strain did not produce chronic infection but caused either acute fatal septicemia or else prompt recovery. A strain of M. pyogenes var. aureus caused the severest and most chronic septic arthritis and pyemic nephritis observed, while an albus strain of Staphylococcus and two alpha streptococci were almost nonpathogenic for the rabbit.

A Group A Type 12 Streptococcus isolated from a patient with acute nephritis did not produce nephritis of a different nature in rabbits than did other Group A strains.

A few instances of carditis somewhat resembling that seen in rheumatic fever were observed in rabbits having infections with both streptococci and a naturally occurring disease. This disease was seen in one-third of the animals and was partly characterized by some cardiovascular inflammation. The question of synergistic action among infectious agents in the pathogenesis of rheumatic fever is considered.

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Case Reports

SARCOIDOSIS ASSOCIATED WITH GLOMERULONEPHRITIS

PELAYO CORREA, M.D.
ATLANTA

THE OCCURRENCE of renal calcinosis and nephrolithiasis in patients with sarcoidosis has been emphasized in several recent publications.* Extensive involvement of the kidneys by sarcoid is not commonly seen at autopsy and does not usually account for the clinical evidence of disturbed renal function.[1] For these reasons the renal complications which are sometimes associated with sarcoidosis are attributed to the effects of hypercalcemia on the kidney. It seems, therefore, of interest to record the findings in a patient with long standing and widespread sarcoidosis who died with renal failure and uremia subsequent to subacute glomerulonephritis.

REPORT OF A CASE

A 37-year-old Negro woman was first admitted to the Grady Memorial Hospital on March 4, 1948, complaining of a generalized skin rash of five months' duration, weight loss, night sweats, and mild exertional dyspnea. She had spent the first 15 years of her life in rural South Georgia and had since been employed at various times as a domestic, a clerk, and a welder. The skin rash began as a "knot" in the left leg and rapidly spread to involve all extremities, the face, and the back. She had received oxophenarsine hydrochloride (Mapharsen) and bismuth therapy after a positive serologic test for syphilis in 1944.

Physical examination showed a patient of normal development and nutrition. Temperature and pulse rate were normal. The blood pressure was 107 mm. of mercury systolic and 68 mm. diastolic. Multiple well-defined, shiny, slightly hyperpigmented areas varying in size from 1 to 2 cm. were present over the face, extremities, abdomen, and back. Some of the lesions were desquamating. Both lacrimal glands were studded with multiple white, pinpoint-sized lesions. A few similar lesions were seen on the tonsils, which were markedly hypertrophied. The parotid glands had a finely granular consistency. There was marked generalized lymphadenopathy. Fine rales were heard at both lung bases and there was diminished tactile fremitus at the right base. The cardiac rhythm was regular, and no murmurs were heard. The liver was slightly enlarged.

The specific gravity of the urine varied between 1.005 and 1.026. There was 1 + albuminuria and 1 or 2 granular casts per high power field. The blood erythrocyte count was 4,320,000 per cubic millimeter, and the blood hemoglobin content was 12.0 gm. per 100 cc. The white blood cell count was 7,750 per cubic millimeter, with 76% polymorphonuclear leucocytes, 11% lymphocytes, 10% monocytes, and 3% eosinophiles. The blood nonprotein nitrogen concentration was 49 mg. per 100 cc. The total protein content of the plasma was 8.2 gm. per 100 cc., with 3.4 gm. of albumin and 4.8 gm. of globulin. The serum calcium content was 11.6 mg. per 100 cc., and the content of inorganic phosphorus was 3.6 mg. per 100 cc. Phenolsulfonephthalein excretion was 50% in two hours. The Kahn test for syphilis and the second strength tuberculin skin test (PPD) were negative. A roentgenogram of the chest revealed a diffuse increase in pulmonary

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* References 1 and 2.

markings with numerous confluent soft tissue lesions in both lung fields; no cavitation or pleural effusion was present; there was bilateral hilar adenopathy with some widening of the mediastinum to the right. The heart was normal.

Biopsy of cutaneous lesions and of an axillary lymph node showed numerous florid sarcoid granulomata without significant fibrosis (Fig. 1) and confirmed the clinical impression of sarcoidosis. The patient received a total dose of 30 mg. of dihydrotachysterol during her hospital stay, with some improvement of the cutaneous lesions but no change in the radiologic picture of the chest.

The patient was next seen in the eye clinic during May, 1950, because of corneal ulceration. This responded satisfactorily to conservative treatment.

The patient was again admitted to the hospital in June, 1951, because of exertional dyspnea of two weeks' duration, orthopnea, right anterior pleuritic chest pain, and cough productive of yellowish sputum. Her temperature was 101 F., pulse rate 100 beats per minute, and blood pressure 111/70 mm. of mercury. There were moist rales bilaterally and findings of right-sided

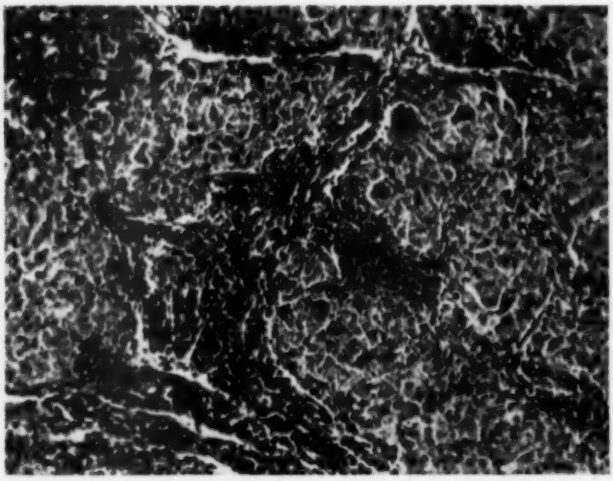

Fig. 1.—Sarcoidosis of lymph node removed five years before death. Florid lesions without fibrosis or necrosis. Hematoxylin and phloxine; × 120.

pleural effusion. The heart had normal sinus rhythm, no murmurs, and an accentuated pulmonic second sound. The liver and spleen were enlarged, and there was slight pedal edema.

Urinalysis revealed 2+ albuminuria; 4 to 8 red blood cells per high power field; 2 to 3 white blood cells, and 30 to 50 granular casts per high power field. The blood hemoglobin content was 13.7 gm. per 100 cc. The white blood cell count was 7,500 per cubic millimeter. Roentgenologic examination of the chest revealed generalized fibrotic changes and a localized confluent infiltration in the right upper lung field. The hilar areas were elevated, with prominent pulmonary arteries. The heart was not enlarged. An electrocardiogram was consistent with right ventricular strain. Cardiac catheterization showed right ventricular hypertension without evidence of right-sided heart failure. Arterial oxygen saturation was 85%, and the cardiac output was considered normal.

The manifestations of congestive heart failure cleared on a regimen of sodium restriction and meralluride (Mercuhydrin). The patient was discharged and remained asymptomatic, except for mild exertional dyspnea. Four months following discharge the patient developed weight gain, bilateral pulmonary rales and pedal edema, for which she was digitalized and given meralluride. Improvement, however, was minimal.

Progressive fluid retention led to a third hospitalization in July, 1952. Physical examination was essentially unaltered except for marked liver enlargement and dependent edema.

The specific gravity of the urine was 1.025 with 2+ albuminuria, occasional white and red blood cells and some granular casts. The total protein content of the plasma was 10 gm. per 100 cc. The serum calcium content was 11.6 mg. and the inorganic phosphorus 3.5 mg. per 100 cc. Roentgenologic examination of the chest showed large cystic areas of rarefaction in the right lung without appreciable change in the left lung field.

After discharge, the patient was free of peripheral edema for seven months, but then fluid began to reaccumulate.

The patient was admitted for the last time in March, 1953. Five days before admission she had stopped eating and taking fluid and had gradually become comatose. She was found to be deeply stuporous and dehydrated. The temperature was 98 F., the pulse rate 100 beats per minute, the respiratory rate 8 per minute, and the blood pressure 90/60 mm. of mercury. The neck veins were markedly distended. There was fluid in the right hemithorax and moist

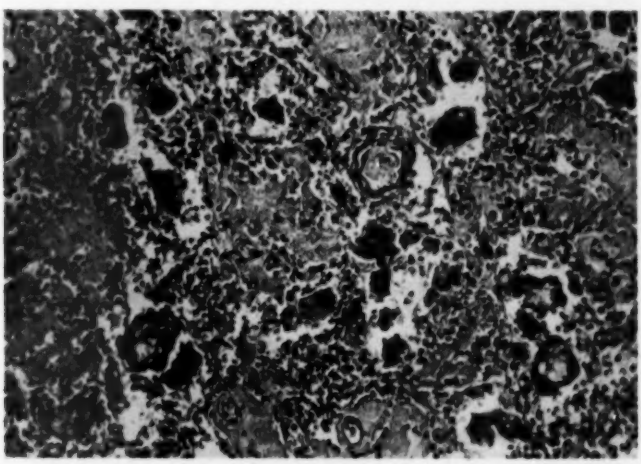

Fig. 2.—Sarcoidosis of lymph node obtained at autopsy showing marked fibrosis and many large Schaumann bodies. Hematoxylin and phloxine; × 120.

inspiratory rales over the left upper lobe. The remainder of the physical examination was unchanged, except for hyperactive deep tendon reflexes. There was no peripheral edema.

The specific gravity of the urine was 1.022. There was 4+ albuminuria and gross hematuria. The hemoglobin content of the blood was 12.2 gm. per 100 cc., and the white blood cell count was 21,300 per cubic millimeter. The blood urea nitrogen was 186 mg. per 100 cc. Roentgenologic examination of the chest revealed decrease in the amount of pleural effusion on the right, with an increase in the degree of infiltration in the right upper lung field.

Despite antibiotic therapy and attempts to improve hydration the patient died 18 hours after admission.

Gross Autopsy Findings.—Autopsy was performed 18 hours after death, and the significant findings were as follows: The spleen was enlarged (320 gm.) and firm in consistency. Its cut surfaces were studded with multiple whitish nodules, which averaged 3 mm. in diameter. The lymph nodes throughout the body were enlarged and were especially prominent in the abdominal and thoracic cavities. They averaged 4 cm. in diameter and were matted together by dense fibrous bands. The cut surfaces were homogeneous and of grayish-white color. There was no evidence of necrosis or calcification. The liver (1,600 gm.) was of normal color, had an indistinct

nodular surface, and showed an increase in consistency. On cut section, however, the lobular architecture did not appear grossly abnormal. The lungs, particularly the left, were markedly enlarged. Dense fibrous adhesions were present over both pleural surfaces, especially prominent in the apical portions. The apices of both lungs revealed marked diffuse fibrosis and ectasia of the bronchi. The lower lobes were crepitant and revealed no visible or palpable nodules. No areas of cavitation or caseation were present. The enlarged hilar lymph nodes slightly compressed the main stem bronchus on each side. The branches of the pulmonary artery showed some atherosclerosis. The heart (225 gm.) revealed hypertrophy of the right ventricle (8 mm.) and dilatation of the chambers on the right side. Ill-defined minute grayish-white areas were seen scattered through the myocardium. The coronary arteries were not remarkable. Multiple depigmented and scarred areas surrounded by a ring of pigmentation diffusely involved the skin.

The kidneys each weighed 200 gm. and revealed a smooth brownish surface. The capsule stripped with ease. On the cut surfaces the general architecture was not altered. No stones were found in the calyces, pelves, or lower urinary tract.

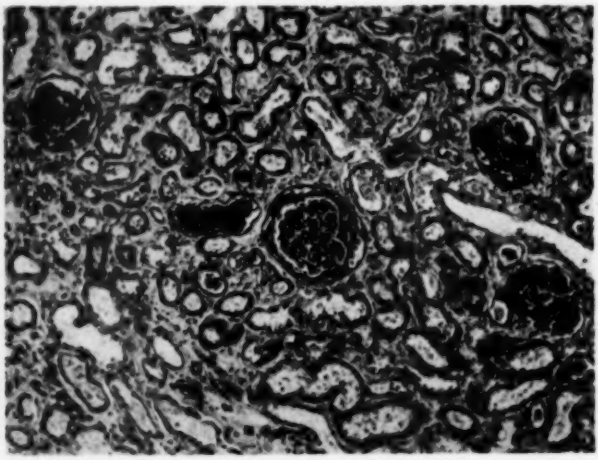

Fig. 3.—Kidney with subacute glomerulonephritis. The tubule to the left of the glomerulus in the center is packed with red blood cells. Hematoxylin and phloxine; × 72.

Incidental findings were right hydrothorax (1000 cc.), a 1 cm. sessile polyp in the cecum, and two endometrial polypi, each measuring 1 cm. in diameter.

Histological Examination.—Typical sarcoid lesions were found in the spleen, lymph nodes, pleura, myocardium, salivary glands, skin, liver, wall of the stomach and colon, meninges of the spinal cord, and thyroid gland (Fig. 2). Most of the lesions showed involution associated with fibrosis but some florid granulomata were present. Numerous asteroids and Schaumann bodies were found. No evidence of caseation necrosis was seen in any of the lesions, and the stains for tubercle bacilli were negative. The lungs showed advanced bronchiectasis with foci of acute bronchopneumonia.

The kidneys revealed diffuse and uniform glomerular changes (Fig. 3). There was extensive adhesion formation between the glomerular loops and the capsule, commonly associated with marked crescent formation (Fig. 4). Some neutrophilic

polymorphonuclear leucocytes were present in the glomeruli. Areas of fibrinoid degeneration were lacking. A few fibrosed and hyalinized glomeruli were found. The tubular epithelium, chiefly in the proximal convoluted tubules, was prominent

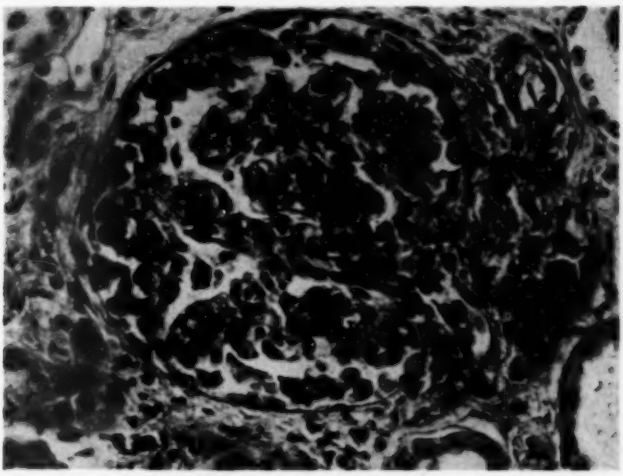

Fig. 4.—Subacute glomerulonephritis with crescent formation and infiltration by some polymorphonuclear leucocytes. Hematoxylin and phloxine; × 276.

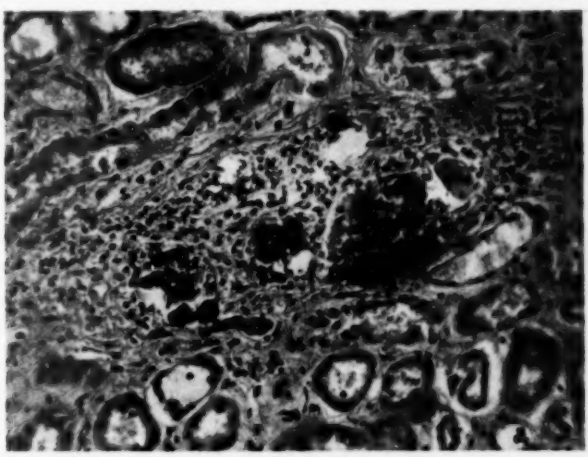

Fig. 5.—Kidney tubules with focal calcium deposits. Hematoxylin and phloxine; × 138.

and revealed dispersion of the protoplasm as well as small hyaline droplets. Numerous casts were found in the tubules. Some of these consisted of red blood cells; others were formed by proteinaceous material. Masses of polymorphonuclear leucocytes were present in some tubules. In some of the loops of Henle and collecting

tubules the epithelium was replaced by a deeply basophilic material which showed a positive reaction for calcium with the van Kossa method. In rare instances areas of calcification were associated with slight chronic inflammatory cell infiltration and fibrosis in the adjoining interstitial connective tissue (Fig. 5). Otherwise this tissue showed only edema. The arteries and arterioles displayed no significant changes. The epithelium and connective tissue of the calyces and pelves were not remarkable. No sarcoid lesions were found in the kidneys.

Final Anatomical Diagnoses.—The final anatomical diagnoses were sarcoidosis, active and healing, involving spleen, lymph nodes, lung, pleura, myocardium, salivary glands, liver, skin, stomach, colon, peritoneum, thyroid gland, and leptomeninges; bronchiectasis; pleural adhesions, fibrous, bilateral; atherosclerosis of branches of pulmonary arteries; cor pulmonale; hydrothorax, right; subacute glomerulonephritis; renal calcinosis, slight; bronchopneumonia, acute; adenomatous polyp of cecum, and endometrial polypi.

COMMENT

The autopsy finding of subacute glomerulonephritis in our patient accounts for her death and correlates well with the clinical manifestations of renal failure and uremia observed during the last admission. From the clinical data it is not possible to determine the time of onset of the glomerulonephritis. While florid granulomata were present in the biopsy specimens obtained five years before death, the sarcoid lesions at autopsy showed advanced healing by fibrosis and a striking increase in asteroid inclusions and Schaumann bodies.

Despite the widespread involvement of most of the organs by sarcoid, the kidneys were free of those lesions. Hypercalcemia was noted in our patient five years before death, but at autopsy only a slight degree of renal calcinosis was present; it probably played no part in the renal failure. The cause of hypercalcemia in patients with sarcoidosis is not known and continues to be a subject of investigation.†

Patients with sarcoidosis and renal failure characterized by hematuria and blood urea nitrogen retention have been reported by Klinefelter [4] and Piaggio Blanco and co-workers.[5] In the case reported by Klinefelter a clinical diagnosis of glomerulonephritis was made. Later the diagnosis of sarcoidosis was established, and the renal disturbances were attributed to sarcoid involvement of the kidneys. Piaggio Blanco and co-workers also attributed the renal findings in their patient to involvement of the kidneys by sarcoid.[6] There were no morphological studies in either case. Other authors,* commenting upon Klinefelter's findings, have suggested that they might be attributed to renal calcinosis. The findings in our patient indicate that sarcoidosis and glomerulonephritis may occur together.

Present-day concepts tend to relate the pathogenesis of both sarcoidosis [7] and glomerulonephritis [8] to a hypersensitivity state, and it would be tempting to speculate about a common denominator for the occurrence of both diseases in our patient. However, a review of the literature and the experience in this department indicate that the association of sarcoidosis and glomerulonephritis has not previously been observed. Therefore the simultaneous occurrence of these two disorders would appear to be coincidental.

† References 2 and 3.

SUMMARY

A middle-aged Negro woman with sarcoidosis of over five years' duration died in renal failure with uremia. Autopsy revealed subacute glomerulonephritis in addition to widespread sarcoidosis. The kidneys showed no evidence of sarcoid granulomata. There was only a slight degree of renal calcinosis. The simultaneous occurrence of sarcoidosis and glomerulonephritis appears to be uncommon and is probably coincidental.

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News and Comment

Institute of Microbiology.—The anniversary of the discovery of streptomycin by Selman A. Waksman, of Rutgers University, a little more than ten years ago will be marked on June 7 with the dedication of the university's new $3,500,000 Institute of Microbiology—a building made possible by the drug. Streptomycin has created a great new industry. Production in one year has nearly reached the $100,000,000 mark. In the United States alone last year nine companies produced between 15,000,000 and 20,000,000 gm. a month. It is also manufactured in two plants in Great Britain; two in Spain; one each in Sweden, Denmark, and Germany; three each in France and Italy; four in Japan, and probably several in countries behind the Iron Curtain.

Royalties from the manufacture of streptomycin have amounted to more than $4,000,000, of which more than 80% has been assigned to the Rutgers Research and Endowment Foundation. It is from these funds that the new building was constructed and equipped. Dr. Waksman long ago laid down the objective of the new Institute: "Particular attention will be devoted to the fundamental aspects of the study of microorganisms, their physiology, their biochemical activities and their relations to higher forms of life, notably man, and to his domesticated animals and plants."

The dedication will be followed by a two-day symposium, June 8 to 9. More than 400 leading scientists from this country and abroad have been invited to this session, which will have as its theme, "Perspectives and Horizons in Microbiology."

Course on Cardiovascular Diseases.—A course in "Newer Developments in Cardiovascular Diseases" will be given at The Mount Sinai Hospital, New York, Oct. 11 through 15, 1954, under the auspices of the American College of Physicians. As the title implies, the recent advances will be stressed. Dr. Arthur M. Master and Dr. Charles K. Friedberg will direct the course, and prominent cardiologists and cardiac surgeons will participate.

Grants.—Grants of $30,000 from the American Cancer Society and $15,000 from the National Cancer Institute of the United States Public Health Service were received recently by the National Committee for Careers in Medical Technology, to be used in a program to recruit more young people into the profession of medical technology.

The announcement of the awards was made by the newly-formed committee, which is sponsored by the American Society of Medical Technologists, the American Society of Clinical Pathologists, and the College of American Pathologists.

Members of the National Committee for Careers in Medical Technology are as follows: Dr. William O. Russell, chairman, representing the American Society of Clinical Pathologists; Miss Ruth Hovde, incoming president of the American Society of Medical Technologists; and Dr. Joseph A. Cunningham, of the College of American Pathologists.

Dr. Esmond R. Long Returns from Spain.—Dr. Esmond R. Long has recently returned to Philadelphia after an extensive trip in Spain, where he visited hospitals and sanatoriums concerned with problems of tuberculosis. He was invited by the Department of Health of the Spanish government and was a representative of the International Union Against Tuberculosis.

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